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(54) Title: USE OF INTERLEUKIN-10 TO PRODUCE A POPULATION OF SUPPRESSOR CELLS

(57) Abstract

Interleukin-10 for producing a population of cells which are capable of inhibiting or suppressing reactions to alloantigens, for example in graft-versus-host disease or tissue rejection, is described. Interleukin-10 for reducing responses in mixed lymphocyte response (MLR) is also described. Exogenous or induced endogenous IL-10 may be used for the inhibition or suppression of the reactions to alloantigens.

USE OF INTERLEUKIN-10 TO PRODUCE
A POPULATION OF SUPPRESSOR CELLS

FIELD OF THE INVENTION

The invention relates generally use of interleukin-10 (IL-10) in producing a population of cells which act to suppress various immunological functions, e.g., methods for treating and inhibiting tissue rejection or other immune functions by administering to an afflicted individual an effective amount of interleukin-10.

BACKGROUND OF THE INVENTION

Interleukin-10 is a cytokine which was originally characterized by its activities in suppressing production of Th1 cytokines. See, e.g., de Vries and de Waal Malefyt (eds. 1995) Interleukin-10 Landes Co., Austin, TX; etc.

Suppression of immunological function finds utility in many different contexts. See, e.g., Paul (ed. 1995) Fundamental Immunology 3d ed., Raven Press, NY. In particular, allogeneic immunity is important in a transplantation context, due largely to its extraordinary strength. As organ and tissue transplants become more common in medical contexts, the ability to minimize problems from tissue rejection exhibit larger economic advantages. In addition, means to minimize autoimmune conditions, to block certain responses to particulate antigens, e.g., bacterial and parasitic, and to minimize reaction to certain soluble antigens, both protein and allergens, will be significant advances for therapeutic purposes.

The lack of fully effective therapeutics to minimize or eliminate tissue rejection, graft vs. host disease, or these other immunological responses leads to many problems. The present invention addresses and provides solutions to many of these problems.

SUMMARY OF THE INVENTION

The invention relates to the use of the cytokine interleukin-10 (IL-10) to suppress the rejection of

5   transplanted tissues. The invention also includes pharmaceutical compositions comprising interleukin-10. Preferably, the interleukin-10 of the invention is selected from the group consisting of the mature polypeptides of the open reading frames defined by the amino acid sequences set

10 forth in SEQ ID NO: 1 and SEQ ID NO: 2. These two forms of IL-10 are sometimes referred to as human IL-10 (or human cytokine synthesis inhibitory factor ("CSIF") and viral IL-10 (or BCRF1), respectively, e.g., Moore, et al. (1990) _Science_ 248:1230-1234; Vieira, et al. (1991) _Proc. Nat'l Acad. Sci. USA_

15 88:1172-1176; Fiorentino, et al. (1989) _J. Exp. Med._ 170:2081-2095; and Hsu, et al. (1990) _Science_ 250:830-832. A homolog has also been described in equine herpesvirus type 2 (Roe, et al. (1993) _Virus Genes_ 7:111-116) as well a numerous counterparts from various species. More preferably, the mature

20 IL-10 used in the method of the invention is IL-10 defined by SEQ ID NO: 3 or IL-10 defined by SEQ ID NO: 4.

Thus, in particular embodiments, the present invention provides a method of reducing or inhibiting graft vs. host disease in a bone marrow transfer in a mammal, comprising

25 administering to the mammal an effective amount of interleukin-10. It also provides a method of inhibiting, by an immune system, an antigen-specific response to subsequent presentation of said antigen, comprising administering to said immune system an effective amount of exogenous interleukin-10 and that

30 antigen. In preferred embodiments, the immune response is mediated by a macrophage, APC, langerhans cell, or dendritic cell; the method further inhibits proliferative response of $CD4^+$ host-reactive T cell clones; or the inhibiting persists for at least about 21 days. In other preferred embodiments,

35 the effective amount is sufficient to decrease responder T cell activation; or may further comprise reduced stimulatory capacity of peripheral blood mononuclear cells, dendritic cells, monocytes, and/or normal B cells.

In another embodiment, the invention provides a
substantially pure antigen-specific anergic T cell
characterized by production upon restimulation of low IL-2; low
IL-4; low IL-5; intermediate IFN-γ; low GM-CSF; and high IL-10;
5   with the population made by administering to precursors of said
T cell with a combination of exogenous IL-10 and antigen. In
preferred embodiments, the precursors are CD4$^+$ T cells; the
cells further produce high TNF-α; the cells induce an anergic
response to the antigen; the administered IL-10 is human IL-10;
10   the IL-10 is administered for at least about 7 days; and/or the
anergic condition persists for at least about 21 days. The
antigen specificity may be to an antigen is selected from a
protein antigen; a particulate antigen; an alloantigen; or an
autoantigen.

15   Another embodiment is a substantially pure antigen-
specific anergic T cell characterized by production upon
restimulation of low IL-2; low IL-5; intermediate IFN-γ; low
GM-CSF; and high IL-10. Typically, the levels of production of
the cytokines is, for IL-2 less than about 500 pg/ml; for IL-5
20   between about 300 and 3000 pg/ml; for IFN-γ at least about 1000
pg/ml; for GM-CSF between about 300-3000 pg/ml; and for IL-10
at least about 3000 pg/ml. Preferably, the IL-10 level upon
restimulation with anti-CD3 is at least about 5x that of a Th1
cell.

25   The invention also embraces a substantially pure T cell
which exhibits an antigen-specific anergy to an antigen,
including, e.g., where the antigen is an alloantigen or self
antigen; which produces IL-10 upon restimulation with anti-CD3
of at least about 3000 pg/ml; or which exhibits the antigen-
30   specific anergy for at least about 21 days.

In another embodiment, the invention provides a method of
suppressing a response in a T cell to an antigen, by
administering to an immune system containing such cell a
combination of exogenous IL-10 and either antigen or anti-CD3
35   antibodies. Preferably, the antigen is alloantigen or self
antigen; but is usually restricted by MHC molecules. In other
embodiments, the method is performed in vivo; or further
suppresses response to subsequent stimulation, e.g., a response
which accompanies tissue transplantation such as an organ or

4

bone marrow transplant. Typically, the T cell is from the
recipient of said tissue transplantation and the antigen is
from the donor MHC. Often, when the response accompanies
tissue transplantation, the administering is prior to the
5   tissue transplantation; the T cell is introduced to the
recipient; or IL-10 is administered to the tissue to be
transplanted before the transplantation, e.g., to the donor
and/or during transport. In other embodiments, the antigen
causes an autoimmune disease.

10   In other embodiments, the invention also provides a method
of suppressing a subsequent response in a T cell to an antigen
by administering to an immune system a combination of exogenous
IL-10; and either antigen or anti-CD3 antibodies. Preferably,
the IL-10 is administered for at least about 7 days.

15   The present invention further provides a method of
inducing in a T cell anergy to an MHC antigen, by administering
to a precursor to the T cell either exogenous IL-10 and
antigen; or exogenous IL-10 with anti-CD3 antibodies.
Preferably, the administering of IL-10 is for at least about 7
20   days.

Another embodiment which is embraced by the invention is a
composition comprising IL-10 and antigen. The composition may
be a pharmaceutical composition comprising IL-10 and a
pharmaceutically acceptable carrier; the IL-10 may be human IL-
25   10; or the antigen may be alloantigen; self antigen; protein
antigen; or particulate antigen.

                    BRIEF DESCRIPTION OF THE DRAWINGS
         Fig. 1 shows a diagram of the vector pcD(SRα) used for
30   expressing IL-10 in mammalian cells.
         Fig. 2 shows a diagram of the vector TRP-C11 used for
expressing IL-10 in bacteria.
         Figs. 3A-3C show histograms of the effects of endogenous
and exogenous IL-10 on proliferative responses in mixed
35   lymphocyte response. Fig. 3A shows the effects of endogenous
and exogenous IL-10 on proliferative responses in mixed
lymphocyte response (MLR): PBMC donor A X irradiated PBMC donor
B. Fig. 3B shows the effects of endogenous and exogenous IL-10
on proliferative responses in MLR: PBMC donor B X irradiated

PBMC donor A. Fig. 3C shows the neutralization of the inhibitory effects of IL-10 by anti-IL-10 mAb.

Figs. 4A-4D shows histograms of the proliferative responses of purified T cells stimulated with various

5 allogeneic cells, each in the presence of increasing concentrations of IL-10. Fig. 4A shows stimulation of purified T cells with allogeneic irradiated elutriated monocytes. Fig. 4B shows stimulation of purified T cells with positively sorted CD14+ monocytes. Fig. 4C shows stimulation of purified T cells

10 with purified B cells. Fig. 4D shows stimulation of purified T cells with Epstein-Barr virus transformed lymphoblastoid cell line (EBV-LCL).

Fig. 5 shows a histogram of the kinetics of IL-10 on peripheral blood mononuclear cells (PBMC) and allogeneic

15 irradiated PBMC.

Fig. 6 shows a histogram of the effect of IL-10 on IL-2 products in MLR in the presence or absence of the anti-IL-2 receptor α chain mAb BB10.

Figs. 7A-7B show the effect of exogenous IL-2 on the

20 reduced alloantigen-induced proliferative response of stimulated T cells. Fig. 7A shows a graph of the effect of exogenous IL-2 on the reduced alloantigen-induced proliferative response of T cells stimulated with allogeneic irradiated PBMC and induced by IL-10. Fig. 7B shows a graph of the effect of

25 exogenous IL-2 on the reduced alloantigen-induced proliferative response of T cells stimulated with purified allogeneic B cells and induced by IL-10.

Figs. 8A-8B show that IL-10 induces an alloantigen-specific anergy in CD4$^+$ T cells in a MLR. Fig. 8A shows [^{3}H]-

30 TdR incorporation of purified CD4$^+$ T cells cultured for 3 days in medium alone (white bar) or stimulated in a primary MLR with allogeneic purified monocytes (gray bar) in the presence of IL-10 (100 U/ml) (black bar) or the anti-IL-2 receptor a chain mAb BB-10 (hatched bar). Fig. 8B shows three different panels

35 which represent cells that have been preactivated as described in Fig. 8A, kept in culture for 10 days in the presence of IL-10, washed, and restimulated with either medium alone (black bars), allogeneic irradiated PBMC isolated from a third party donor different from that of the monocytes used in the

preactivation step (PBMC-2, cross-hatched bars), allogeneic
irradiated PBMC isolated from the same donor used in the
preactivation step (PBMC-1, gray bars), PBMC-1 plus IL-2 (20
U/ml) (black-hatched bars), or PBMC-1 plus anti-CD28 mAbs (10
5   μg/ml) (hatched bars).

Figs. 9A-9B show IL-10 induces a polyclonal anergy in
CD4^{+}T cells activated with CD3 mAb. Fig. 9A: [^{3}H]-TdR
incorporation of purified CD4^{+} T cells cultured for 3 days in
medium alone (white bar), or stimulated with crosslinked anti-
10   CD3 mAbs (500 ng/ml) (black bar) in the presence of IL-10 (100
U/ml) (cross-hatched bar) or the anti-IL-2 receptor α chain mAb
BB-10 (hatched bar). Fig. 9B show three different portions
which represent cells that have been preactivated as described
in Fig. 9A, kept in culture for 10 days in the presence of IL-
15   10, washed, and restimulated with either medium alone (black
bars), cross-linked anti-CD3 mAbs (gray bars), anti-CD3 mAbs
plus IL-2 (20 U/ml) (cross-hatched bars), anti-CD3 mAbs plus
anti-CD28 mAbs (10 μg/ml) (black-hatched bars), or PMA (1
ng/ml) plus Ca^{2+} ionophore (A23187, 500 ng/ml) (white bars).

20   Fig. 10 shows dose dependent effect of IL-10 on anergy
induction in CD4^{+} T cells. CD4^{+} T cells were activated for 10
days with crosslinked anti-CD3 mAbs in the presence medium
alone, IL-10 (5U/ml), IL-10 (20 U/ml), or IL-10 (100 U/ml), as
indicated under the 4 different panels. After 10 days, cells
25   were collected, washed, and restimulated with medium alone
(white bars), crosslinked anti-CD3 mAbs (black bars),
crosslinked anti-CD3 mAbs plus IL-2 (20 U/ml) (hatched bars),
or PMA plus Ca^{2+} ionophore (black hatched bars).

Fig. 11 shows kinetics of anergy induction by IL-10 in
30   CD4^{+} T cells. CD4^{+} T cells were activated with crosslinked
anti-CD3 mAbs in the absence (white bars) or presence (black
bars) of IL-10 (100 U/ml). After different incubation periods,
ranging from 3 to 10 days as indicated, cells were collected,
washed, and restimulated as indicated under each panel with
35   either medium alone, crosslinked anti-CD3 mAbs, crosslinked
anti-CD3 mAbs plus IL-2 (20 U/ml), crosslinked anti-CD3 mAbs
plus anti-CD28 mAbs (10 μg/ml), or PMA plus Ca^{2+} ionophore.

Fig. 12 shows anergy induced by IL-10 in CD4^{+} T cells is
long lasting. CD4^{+} T cells were activated with crosslinked

anti-CD3 mAbs in the absence (white bars) or presence (black
bars) of IL-10 (100 U/ml) for 10 days. After 10 days, cells
were collected, washed, and cultured in the presence of IL-2 (2
U/ml). Twenty four days later, cells were collected and

5   reactivated with crosslinked anti-CD3 mAbs, crosslinked anti-
CD3 mAbs plus IL-2 (20 U/ml), crosslinked anti-CD3 mAbs plus
anti-CD28 mAbs (10 μg/ml), or PMA plus Ca^{2+} ionophore.

     Fig. 13 shows cytofluorometric analysis of anergic T
cells. $CD4^+$ T cells were activated with crosslinked anti-CD3

10   mAbs in the absence or presence of IL-10 (100 U/ml) for 10 days
and the expression of CD3, CD28, and HLA-DR was analyzed before
and after reactivation with crosslinked anti-CD3 mAbs for 24
hrs. The black line shows control T cells cultured in the
absence of IL-10 and the dotted histograms anergic T cells that

15   were cultured in the presence of IL-10.

     Figs. 14A-14B show IL-2R α chain expression on anergic T
cells. $CD4^+$ T cells were activated with crosslinked anti-CD3
mAbs in the presence or absence of IL-10 (100 U/ml) for 3 days
(Fig. 14A) or 10 days (Fig. 14B) and restimulated with either

20   crosslinked anti-CD3 mAbs alone or with crosslinked anti-CD3
mAbs plus IL-2 (100 U/ml). The expression of the CD25 molecule
was analyzed 24 hr after activation by cytofluorometry. The
black line shows the control isotype and the dotted histogram
the labeling with anti-CD25 mAbs.

25   Figs. 15A-15B show calcium mobilization analysis in
anergic cells. $CD4^+$ T cells were activated with anti-CD3 mAbs
in the absence (Fig. 15A) or presence (Fig. 15B) of IL-10 (100
U/ml) for 10 days and loaded with indo-1/AM. As indicated,
anti-CD3 mAbs (10 μg/ml), goat anti-mouse IgG (1 μg/ml), or

30   Ca^{2+} ionophore (500 ng/ml) were added into the cuvette and the
rise of intracellular Ca^{2+} was analyzed by spectrofluorimetry
and measured by the ratio of emission at 405/485 nm.

     Fig. 16 shows that the proliferative responses of purified
$CD4^+$ T cells that had been rendered anergic following

35   stimulation with crosslinked anti-CD3 mAbs (100 ng/ml) in the
presence of IL-10 (100 U/ml) for 10 days, can be partially
restored following restimulation with very high concentrations
of crosslinked anti-CD3 mAbs. After the 10 day induction
phase, the $CD4^+$ T cells were harvested and centrifuged over

8

Ficoll/hypaque to remove non-viable cells. Next the cells were
collected and washed 3 times with PBS. The CD4$^+$ cells (4 x
10^4/ well) were subsequently restimulated by increasing
concentrations (10 ng to 1 mg/ml) crosslinked anti-CD3 mAbs for
5   3 days.

Fig. 17 shows the proliferative responses of Tr1 clones as
compared to those of Th1 and Th2 clones. The T-cell clones
were maintained by stimulation at biweekly intervals with
irradiated feeder cells and PHA. Resting T-cell clones were
10 collected 12 days after stimulation by feeder cells and PHA,
washed and restimulated with crosslinked anti-CD3 mAbs (100
ng/ml) in the presence or absence of anti-IL-10 mAb (10 µg/ml)
or anti-IL-2 mAb (10 µg/ml). It is shown that the
proliferative responses of the Tr1 clones are very low as
15 compared to those of the Th1 and Th2 clones. In addition, the
proliferative responses of Tr1 clones, in contrast to those of
the control Th1 and Th2 clones, are considerably enhanced by
anti-IL-10 mAbs, but even in the presence of anti-IL-10 mAbs
the response never reached the levels of those of the control
20 Th1 and Th2 clones. The proliferative responses of all types
of T-cell clones, including the Tr1 clones, are completely
inhibited by anti-IL-2 mAbs.

Figs. 18A-18B show that IL-10 production by Tr1 clones
occurs rapidly after activation of the cells. Considerable
25 levels of IL-10 are already produced 16 hrs following
activation of the T-cell clones by combinations of either anti-
CD3 or anti-CD28 mAbs (Fig 18A), or anti-CD3 mAbs + the phorbol
ester PMA (Fig 18B). In contrast, only low levels of IL-10
production by Th1 or Th2 clones can first be measured 24 hrs
30 after activation of the cells.

Fig. 19 shows development of murine Tr1 cells in the
presence of IL-10. Naive (MEL14 bright) CD4$^+$ T cells from mice
transgenic for the DO11.10 αβ TCR on a BALB/c genetic
background (Murphy, et al. (1990) Science 250:1920-1722) were
35 stimulated with OVA peptide (0.6 µM) and irradiated splenic
APCs. In addition, the cultures received cytokines: IL-4 (200
U/ml) or IL-10 (100 U/ml) or both. The stimulation, under the
same conditions as above, was repeated weekly for three
consecutive weeks (Murphy, et al. (1996) J. Exp. Med. 183:901-

13). Cells were then harvested, washed, and restimulated with
crosslinked anti-CD3 and anti-CD28 mAbs for 4 hrs at 37° C,
with Brefeldin A (10 µg/ml) added for the last 2 hrs as
described. Openshaw, et al. (1995) J. Exp. Med. 182:1-11. The
5   cells were then fixed and stained for detection of
intracellular cytokine synthesis with directly conjugated FITC
or PE antibodies specific for IFN-γ, IL-2, IL-4, and IL-10, or
a control isotype mAb (CT isotype), as indicated. Cells were
also stained with the anti-TCR clonotype specific mAb KJ1-26
10   (KJ), to show that after 3 weeks of culture all cells were
positive for the transgene.

Fig. 20A and 20B show proliferative responses of Tr1
cells. Fig 20A: The human Th0 (JDV 305) and Tr1 (JDV24)
alloantigen-specific CD4+ T cell clones or the non antigen-
15   specific Tr1-cell clones (JDV15 and JDV 308) were stimulated
with either allogeneic irradiated monocytes for 5 days or with
crosslinked anti-CD3 and anti-CD28 mAbs for 3 days. Fig 20B:
Naive (MEL14 bright) CD4+ T cells from mice transgenic for the
D011.10 αβ TCR on a BALB/c genetic background were
20   differentiated in vitro as described in Figure 19 and
restimulated with OVA peptide (1 µM) and irradiated splenic
APCs for 3 days. One OVA specific Th0 clone (A-7) and two OVA
specific Tr1 type clones (A-10-9 and A-10-11) were stimulated
under the same conditions. The proliferative responses were
25   measured by ^{3}[H]-TdR incorporation during the last 12 hrs of
culture. Cultures of human or murine cells were carried out in
the presence of a control isotype mAb (20 µg/ml- black bars), a
blocking anti-IL-10 mAb (12G8-5 µg/ml for human cells or JES6-
2A5-10 µg/ml for mouse cells - white cross hatched bars), a
30   blocking anti-TGF-β (Genzyme, MA; 10 µg/ml - black cross
hatched bars), or a combination of the two antibodies (white
bars).

Figs 21A and 21B show Tr1 cells inhibit the Ag-specific
proliferative responses of naive CD4+ T cells. Fig 21A:
35   Resting purified human CD4+ T cells from the donor JDV were
stimulated by allogeneic purified irradiated monocytes for 5
days in the bottom compartment of a transwell well. The
resting T cells were co-cultured with different autologous
clones contained in the transwell basket, either an

alloantigen-specific Tr1 clone (JDV24), an alloantigen-specific
Th0 clone (JDV305), or a non antigen-specific Tr1 clone (JDV
308). After 5 days the basket was removed and the
proliferative response of the resting CD4$^+$ T cells was analyzed
by ^{3}[H]-TdR incorporation during the last 12 hrs of the
culture. Fig 21B: Naive (MEL14 bright) CD4$^+$ T cells from mice
transgenic for the D011.10 αβ TCR on a BALB/c genetic
background were stimulated in the bottom compartment of a
transwell system with OVA peptide (1 μM) and irradiated APCs
for 3 days. These naive T cells were co-cultured with the
indicated populations contained in the transwell basket: OVA-
specific CD4$^+$ T cell populations differentiated in vitro by
three consecutive restimulations in the presence of IL-4 (IL-4
Diff.), IL-10 (IL-10 Diff.) or IL-4 and IL-10 (IL4/10 Diff.)
respectively or OVA specific CD4$^+$ T cell clones A-10-9, A-10-11
(Tr1 type clones), or A-7 (Th1 type clone). After 3 days the
basket was removed and the proliferative response of the naive
CD4$^+$ T cells was analyzed by ^{3}[H]-TdR incorporation during the
last 12 hrs of the culture. Cultures of human and murine cells
were carried out in the presence of a control isotype (20
μg/ml- black bars), a blocking anti-IL-10 mAb (12G8-5 μg/ml for
human cells or JES6-2A5-10 μg/ml for mouse cells-white cross
hatched bars), a blocking anti-TGFβ (Genzyme, MA- 10μg/ml-
black cross hatched bars), or a combination of the two
antibodies (white bars).

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed, in certain embodiments, to
methods of using IL-10 to suppress tissue rejection in
transplant patients. The invention also includes
pharmaceutical compositions comprising IL-10 for carrying out
the method. Various IL-10s for use in the invention are
selected from the group of mature polypeptides encoded by the
open reading frames defined by the cDNA inserts of pH5C, pH15C,
and pBCRF1(SRα), which were deposited with the American Type
Culture Collection (ATCC), Rockville, Maryland, on December 20,
1989, under accession numbers 68191, 68192, and 68193.
Constructs can also be based upon other expression systems.
See, e.g., Pouwels, et al. (1985 and Supplements) Cloning

Vectors: A Laboratory Manual Elsevier, N.Y.; and Rodriquez, et
al. (1988; eds.) Vectors: A Survey of Molecular Cloning Vectors
and Their Uses Buttersworth, Boston, MA. PCR methods may be
applied to isolate other genes encoding IL-10s, e.g.,
5   polypmorphic or species variants.

       IL-10 is a cytokine with potent immunosuppressive
properties. See, e.g., Mosmann, et al., U.S. Patent No.
5,231,012, which is incorporated herein by reference. IL-10
inhibits antigen-specific T-cell proliferation at different
10   levels. IL-10 inhibits the antigen-presenting and accessory
cell function of professional antigen-presenting cells such as
monocytes, dendritic cells and Langerhans cells by
downregulation of the expression of MHC class II molecules and
of the adhesion and co-stimulatory molecules ICAM-1 and B7.1
15   and B7.2 (reviewed in de Vries and de Waal Malefyt (1995; eds.)
Interleukin 10 Landes Co, Austin TX). IL-10 also inhibits IL-
12 production by these cells, which promotes T-cell activation
and the differentiation of Th1 cells (D'Andrea, et al. (1993)
J. Exp. Med. 178:1041; Hsieh, et al. (1993) Science 260:547.
20   In addition, IL-10 directly inhibits T-cell proliferation by
inhibiting IL-2 gene transcription and IL-2 production by these
cells (reviewed in de Vries and de Waal Malefyt (1995; eds.)
Interleukin 10 Landes Co, Austin TX).

       Moreover, IL-10 has strong anti-inflammatory properties.
25   It inhibits the production of the pro-inflammatory cytokines
TNF-α, IL-1α, IL-1β, IL-6, and chemokines such as IL-8, MIP-
1α, and MIP-1β by activated monocytes/ macrophages,
neutrophils, eosinophils, and mast cells (reviewed in de Vries
and de Waal Malefyt (1995; eds.) Interleukin 10 Landes Co,
30   Austin TX; Takanashi, et al. (1994) J. Exp. Med. 180:711;
Arock, et al. (1996) Eur. J. Med. 26:166). In addition, IL-10
supports nitric oxide-dependent microbicidal activity of
macrophages and prostaglandin production by these cells
(Gazzinelli, et al. (1992) J. Immunol. 148:1792; Cunha, et al.
35   (1992) Biochem. Biophys. Res. Commun. 182:1155; Niiro, et al.
(1994) Int. Immunol. 6:661. On the other hand, it upregulates
the production of the IL-1 receptor antagonist by monocytes and
granulocytes (reviewed in de Vries and de Waal Malefyt (1995;
eds.) Interleukin 10 Landes Co, Austin TX).

Collectively, these data indicate that IL-10 may have
potential clinical utility in the treatment of diseases
associated with undesired T-cell activation and T-cell
expansion such as autoimmune diseases, organ and bone marrow

5   transplant rejection, graft-versus-host disease, parasitic
infections, e.g., granulomas, inflammatory diseases, e.g.,
Crohn's disease, colitis, pancreatitis, inflammatory lung and
eye diseases, parasitic diseases, and allergic diseases, e.g.,
asthma, atopic dermatitis, and rhinitis.

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I.   Assays for Interleukin-10

IL-10s exhibit several biological activities which could
form the basis of assays and units. See, e.g., Coligan (1989
and periodic supplements; ed.) Current Protocols in Immunology

15   Greene/Wiley, NY. In particular, IL-10s have property of
inhibiting the synthesis of at least one cytokine in the group
consisting of IFN-γ, lymphotoxin, IL-2, IL-3, and GM-CSF in a
population of T helper cells induced to synthesize one or more
of these cytokines by exposure to antigen and antigen

20   presenting cells (APCs). In this activity, the APCs are
treated so that they are incapable of replication, but that
their antigen processing machinery remains functional. This is
conveniently accomplished by irradiating the APCs, e.g., with
about 1500-3000 R (gamma or X-radiation) before mixing with the

25   T cells.

Alternatively, cytokine inhibition may be assayed in
primary or, preferably, secondary mixed lymphocyte reactions
(MLR), in which case syngeneic APCs need not be used. MLRs are
well known in the art, e.g., Bradley, pgs. 162-166, in Mishell,

30   et al. (1980; eds.) Selected Methods in Cellular Immunology
Freeman, San Francisco; and Battisto et al. (1987) Meth. in
Enzymol. 150:83-91 Academic Press. Briefly, two populations of
allogeneic lymphoid cells are mixed, one of the populations
having been treated prior to mixing to prevent proliferation,

35   e.g., by irradiation. Preferably, the cell populations are
prepared at a concentration of about 2 x 10^6 cells/ml in
supplemented medium, e.g., RPMI 1640 with 10% fetal calf serum.
For both controls and test cultures, mix 0.5 ml of each
population for the assay. For a secondary MLR, the cells

remaining after 7 days in the primary MLR are re-stimulated by
freshly prepared, irradiated stimulator cells. The sample
suspected of containing IL-10 may be added to the test cultures
at the time of mixing, and both controls and test cultures may
5 be assayed for cytokine production from 1 to 3 days after
mixing.

Obtaining T cell populations and/or APC populations for
IL-10 assays employs techniques well known in the art which are
fully described, e.g., in DiSabato, et al. (1984; eds.) Meth.
10 in Enzymol. vol. 108 Academic Press. APCs for the preferred
IL-10 assay are peripheral blood monocytes. These are obtained
using standard techniques, e.g., as described by Boyum (1984)
Meth. in Enzymol. 108:88-102; Mage (1984) Meth. in Enzymol.
108:118-132; Litvin, et al. (1984) Meth. in Enzymol. 108:298-
15 302; Stevenson (1984) Meth. in Enzymol. 108:242-249; and
Romain, et al. (1984) Meth. in Enzymol. 108:148-153, which
references are incorporated by reference. Preferably, helper
T cells are used in the IL-10 assays, which are obtained by
first separating lymphocytes from the peripheral blood then
20 selecting, e.g., by panning or flow cytometry, helper cells
using a commercially available anti-CD4 antibody, e.g., OKT4
described in U.S. Patent No. 4,381,295 and available from Ortho
Pharmaceutical Corp. The requisite techniques are fully
disclosed in Boyum (1968) Scand. J. Clin. Lab. Invest.
25 21(Suppl. 97):77; Meth. in Enzymol. Vol. 108 (cited above), and
Bram, et al. (1986) Meth. in Enzymol. 121:737-748. Generally
PBLs are obtained from fresh blood by Ficoll-Hypaque density
gradient centrifugation.

A variety of antigens can be employed in the assay, e.g.,
30 keyhole limpet hemocyanin (KLH), fowl γ-globulin, or the like.
More preferably, in place of antigen, helper T cells are
stimulated with anti-CD3 monoclonal antibody, e.g., OKT3
disclosed in U.S. Patent No. 4,361,549, in the assay.

Cytokine concentrations in control and test samples are
35 measured by standard biological and/or immunochemical assays.
Construction of immunochemical assays for specific cytokines is
well known in the art when the purified cytokine is available,
e.g., Campbell (1984) Monoclonal Antibody Technology Elsevier,
Amsterdam; Tijssen (1985) Practice and Theory of Enzyme

Immunoassays Elsevier, Amsterdam; and U.S. Patent No. 4,486,530
are exemplary of the extensive literature on the subject.
ELISA kits for human IL-2, human IL-3, and human GM-CSF are
commercially available from Genzyme Corp. (Boston, MA); and an
5   ELISA kit for human IFN-γ is commercially available from
Endogen, Inc. (Boston, MA). Polyclonal antibodies specific for
human lymphotoxin are available from Genzyme Corp. which can be
used in a radioimmunoassay for human lymphotoxin, e.g., Chard
(1982) An Introduction to Radioimmunoassay and Related
10   Techniques Elsevier, Amsterdam. See also Coligan, et al.
(eds.) Current Protocols in Immunology.

        Biological assays of the cytokines listed above can also
be used to determine IL-10 activity. A biological assay for
human lymphotoxin is disclosed in Aggarwal (1985) Meth. in
15   Enzymol. 116:441-447, and Matthews, et al., pgs. 221-225, in
Clemens, et al. (1987; eds.) Lymphokines and Interferons: A
Practical Approach IRL Press, Washington, D.C. Human IL-2 and
GM-CSF can be assayed with factor dependent cell lines CTLL-2
and KG-1, available from the ATCC under accession numbers TIB
20   214 and CCL 246, respectively. Human IL-3 can be assayed by
its ability to stimulate the formation of a wide range of
hematopoietic cell colonies in soft agar cultures, e.g., as
described by Metcalf (1984) The Hemopoietic Colony Stimulating
Factors Elsevier, Amsterdam. INF-γ can be quantified with
25   anti-viral assays, e.g., Meager, pgs. 129-147, in Clemens, et
al., eds. (cited above).

        Cytokine mRNA production can be measured and analyzed by
cytoplasmic dot hybridization as described by White, et al.
(1982) J. Biol. Chem. 257:8569-8572; and Gillespie, et al.,
30   U.S. Patent No. 4,483,920, which are each incorporated herein
by reference. Other approaches include dot blotting using
purified RNA, e.g., chapter 6, in Hames, et al. (1985; eds.)
Nucleic Acid Hybridization A Practical Approach IRL Press,
Washington, D.C.

35   In some cases, samples to be tested for IL-10 activity may
be pretreated to remove predetermined cytokines that might
interfere with the assay. For example, IL-2 increases the
production of IFN-γ in some cells. Thus, depending on the
helper T cells used in the assay, IL-2 should be removed from

the sample being tested. Such removals are conveniently
accomplished by passing the sample over a standard anti-
cytokine affinity column.

For convenience, units of IL-10 activity are defined in
terms of the ability of IL-10 to augment the IL-4-induced
proliferation of MC/9 cells, which are described in U.S. Patent
No. 4,559,310 and available from the ATCC under accession
number CRL 8306. 1 unit/ml is defined as the concentration of
IL-10 which gives 50% of maximum stimulation of MC/9
proliferation above the level of IL-4 in the following assay.
Prepare multiple duplicate dilutions of IL-4 and IL-10 in 50 μl
of medium per well in a standard microtiter plate. For
example, medium consists of RPMI 1640, 10% fetal calf serum, 50
μM 2-mercaptoethanol, 2 mM glutamine, penicillin (100 U/L) and
streptomycin (100 μg/L). Add IL-4, 25 μl/well of 1600 U/ml
(400 U/ml final) diluted in medium and incubate overnight,
e.g., 20-24 hours. ^{3}H-thymidine (e.g., 50 μCi/ml in medium) is
added at 0.5-1.0 μCi/well and the cells are again incubated
overnight, after which cells are harvested and incorporated
radioactivity measured.

II.   Purification and Pharmaceutical Compositions

When polypeptides of the present invention are expressed
in soluble form, for example as a secreted product of
transformed yeast or mammalian cells, they can be purified
according to standard procedures of the art, including steps of
ammonium sulfate precipitation, ion exchange chromatography,
gel filtration, electrophoresis, affinity chromatography,
and/or the like, e.g., "Enzyme Purification and Related
Techniques," (1977) Methods in Enzymology 22:233-577, and
Scopes (1982) Protein Purification: Principles and Practice
Springer-Verlag, New York; provide guidance in such
purifications. Likewise, when polypeptides of the invention
are expressed in insoluble form, for example as aggregates,
inclusion bodies, or the like, they can be purified by standard
procedures in the art, including separating the inclusion
bodies from disrupted host cells by centrifugation,
solubilizing the inclusion bodies with chaotropic and reducing
agents, diluting the solubilized mixture, and lowering the

concentration of chaotropic agent and reducing agent allowing
the polypeptide to take on a biologically active conformation.
The latter procedures are disclosed in the following
references, which are incorporated by reference: Winkler, et
5   al. (1986) Biochemistry 25:4041-4045; Winker, et al. (1985)
Biotechnology 3:992-998; Koths, et al., U.S. Patent No.
4,569,790; and European patent applications 86306917.5 and
86306353.3. Recombinant methods allow production of proteins
with either fusion products, which allow for purification, or
10   epitope tags, which also assist in purification or detection.
Specific means may be engineered into the construct to allow
for removal of extraneous sequences. It is believed that the
amino terminus of the IL-10 molecule is important for binding
to its receptor, so it will usually be preferred to make
15   fusions at the carboxy terminus.

As used herein, "effective amount" means an amount
sufficient to provide the desired result, e.g., in an
appropriate context, to reduce or prevent tissue rejection.
Preferably, such amount will have minimal negative side
20   effects. The effective amount for a particular patient may
vary depending on such factors as the state, type, and amount
of tissue transplanted, the overall health of the patient,
method of administration, the severity of side-effects, and the
like. Generally, IL-10 is administered as a pharmaceutical
25   composition comprising an effective amount of IL-10 and a
pharmaceutical carrier. A pharmaceutical carrier can be any
compatible, non-toxic substance suitable for delivering the
compositions of the invention to a patient. Generally,
compositions useful for parenteral administration of such drugs
30   are well known, e.g., Remington's Pharmaceutical Science Mack
Publishing Company, Easton, PA; or Gilman, et al. (eds.)
Goodman and Gilman's: The Pharmacological Bases of Therapeutics
Permagon Press. Alternatively, compositions of the invention
may be introduced into a patient's body by implantable or
35   injectable drug delivery system, e.g., Urquhart, et al. (1984)
Ann. Rev. Pharmacol. Toxicol. 24:199-236; Lewis (1981; ed.)
Controlled Release of Pesticides and Pharmaceuticals Plenum
Press, New York; U.S. Patent No. 3,773,919; U.S. Patent No.
3,270,960; and the like.

When administered parenterally, the IL-10 is preferably
formulated in a unit dosage injectable form (solution,
suspension, emulsion) in association with a pharmaceutical
carrier. See, e.g., Avis, et al. (1993; eds.) Pharmaceutical

5   Dosage Forms: Parenteral Medications Dekker, NY; Lieberman, et
al. (1990; eds.) Pharmaceutical Dosage Forms: Tablets Dekker,
NY; and Lieberman, et al. (1990; eds.) Pharmaceutical Dosage
Forms: Disperse Systems Dekker, NY. Examples of such carriers
include normal saline, Ringer's solution, dextrose solution,

10 and Hank's solution. Nonaqueous carriers such as fixed oils
and ethyl oleate may also be used. A preferred carrier is 5%
dextrose/saline. The carrier may contain minor amounts of
additives such as substances that enhance isotonicity and
chemical stability, e.g., buffers and preservatives. The IL-10

15 is preferably formulated in purified form substantially free of
aggregates and other proteins at a concentration in the range
of about 5 to 20 μg/ml. Preferably, IL-10 is administered by
continuous infusion so that an amount in the range of about 50-
800 μg is delivered per day (i.e., about 1-16 μg/kg/day). The

20 daily infusion rate may be varied based on monitoring of side
effects and blood cell counts.

III. Tr1 Cells

The present invention also provides antigen-specific
25 anergic T cells, which fail to respond in an antigen-specific
fashion to representation of the antigen. See, e.g., Paul
(1993; ed.) Fundamental Immunology Raven Press. This antigen-
specific anergy can be distinguished from generic tolerance in
that many forms of tolerance result from blockage of the T cell

30 receptor signal at the cell surface and thus will be
independent of the antigen. The antigen-specific anergy
described here, as shown below, does not appear to affect the
Ca^{++} dependent signaling pathway, e.g., the Ras/Raf/MAPK
pathway, of signal transduction, since the T cell receptor and

35 the Ca^{++} flux responses to antigen engagement remain.
Moreover, the anergy does not require constant administration
of an agent to block the early stages of signal transduction,
e.g., necessary to actively block T cell receptor function.
The anergy seems to be maintained for a period of time, e.g.,

for at least about 14 days, 18 days, 21 days, 24 days, etc.   It
may remain for weeks, months, and preferably years.

The antigen-specific anergy described herein can be
produced by presenting a combination of IL-10 with antigen.
5   The components are presented to the immune system, or cells
thereof, for adequate periods of time, often completely
coextensive, though the period may not necessarily require both
components for the entire duration.   This period will typically
be at least about 5 days, more typically at least about 7 days,
10   preferably at least about 9-11 days, and more preferably at
least about 13-15 days or more.   The dosing of the IL-10 and or
antigen may depend on various factors, including, e.g., the
antigen, the duration of the periods, whether both are present,
whether the IL-10 is presented before antigen, etc.
15   Preferably, both components are presented for at least about 7
days.

IL-10 has been described above.   Other means to effect
higher IL-10 levels include stimulation of endogenous IL-10,
including, e.g., LPS, TNF-α, IL-12, BCG1 (Bacillus Calmett
20   Guerin), Corynebacterium parvus, poly I-C (alloadjuvant for
activating monocytes and macrophages), etc.   Agonistic
antibodies to the IL-10 receptor may also function as an
agonist.

Various types of antigens exist for which antigen-specific
25   T cell anergy may be important.   Both alloantigens and self
antigens are presented in the context of MHC.   See, e.g., Paul
(ed.) Fundamental Immunology.   Other antigens for which T cell
anergy may be important include soluble antigens, e.g., soluble
proteins or fragments of insoluble complexes, particulate
30   antigens, e.g., bacteria or parasites, and allergens.   Various
forms of antigen will be presented with IL-10 to induce
antigen-specific antigen, as described.

Antigen-specific anergy involves a mechanism which is
distinguishable from certain other form: of non-responsiveness.
35   These Tr1 (which had in the priority filing been designated Th3
cells, but have been redesignated Tr1 cells herein, to
distinguish from another population of cells described by
Weiner, et al. (1996) Faseb Meeting, New Orleans, Louisiana. A
2558) cells appear to be memory T cells, e.g., CD45+, in

contrast to naive T cells, e.g., CD45RA+ or CD45RO-. In
particular, this antigen-specific anergy reflects the inability
of these antigen-specific cells to respond to subsequent
restimulation with the specific antigen, typically with IL-2.

5   When antigen-specific anergic cells are restimulated with IL-2
and antigen at normal levels, the cells fail either to
proliferate significantly, or to produce the cytokines.
However, the anergic population containing Tr1 cells, when
stimulated in a subsequent presentation with anti-CD3

10   antibodies (at about 100 ng/ml) will stimulate only the growth
of the Th0, Th1, and Th2 T cells. Analysis of the cytokine
production by these defined T helper subset cells is well known
when stimulated at the higher levels of anti-CD3 of about 1-10
μg/ml. However, at this level of stimulation, the Tr1 cells

15   seem not to be stimulated to proliferate or produce cytokines.
The Tr1 cells seem to be stimulated both to proliferate and
produce cytokines with anti-CD3 antibodies at about 100-700
μg/ml. Thus, if the cells in a mixed population are diluted
out to single cells in wells, all of the T helper subsets will

20   be stimulated with anti-CD3 antibodies at the 100-700 μg/ml,
which allows evaluation of the cytokine production profiles of
the different subsets Th0, Th1, Th2, and Tr1, as described.

       The response to subsequent anti-CD3 antibody general
stimulation, e.g., through the T cell receptor/Cd3 complex, can

25   be quantitated by various methods. As described above, various
cytokines may be measured according to biological activity.
Preferably, a quantitation of accumulated protein may be
determined by various immuno- or other assays, as described
below. Alternatively, mRNA production may be measured to

30   establish levels of stimulation of transcription.

       Typically, cytokines are measured after accumulation of
secreted protein over set periods of time upon subsequent,
e.g., secondary or subsequent, stimulation using anti-CD3
antibody (100-700 μg/ml). Thus, the time for accumulation is

35   preferably at least about 24 h in a volume of about 1 ml, but
may be longer, and may include other feeder cell layers, etc.

       Cell proliferation after subsequent stimulation can be
measured by standard methods. Often this includes measuring

incorporation of nucleotides, but may also involve measuring cell numbers, cell volumes, etc.

While certain responses of tolerance characterized as anergy result from blockage of signaling at the T cell receptor
5   (see Weiss and Littman (1994) Cell 76:263-274; Chan, et al. (1994) Ann. Rev. Immunol. 12:555-592; and Fraser, et al. Immunol. Today 14:357-362), the anergy described herein exists with functional T cell receptor. In particular, stimulation with anti-CD3 still results in a Ca^{++} flux. But the antigen-
10   specific T cells do not respond to the antigen stimulation in the normal manner, e.g., by production of cytokines and/or cell proliferation.

The anergy provided herein involves either a much lowered proliferative responsiveness to IL-2 and/or antigen, e.g., less
15   than about 50% response, usually less than about 40% response, more usually less than about 30% response, preferably less than about 20%, and more preferably 10%, or less as compared to non-anergic cells. Alternatively, the stimulation required to induce a response requires much larger amounts of IL-2 and
20   antigen, e.g., 5-50x, to elicit the equivalent proliferative response. The anergy also is reflected in a different cytokine production profile upon restimulation of clones with anti-CD3 antibodies. See below.

The measure of cytokine production is after restimulation
25   of clones with the specific antigen, although more usually after generic stimulation with anti-CD3 (in vitro at about 10 µg/ml or more), which apparently activates through the T cell receptor. This stimulation results in a much attenuated cellular proliferation response, and a distinguishable cytokine
30   production profile. Among the notable differences in cytokine production after restimulation and cloning are undetected IL-4 and high IL-10 production. See below, e.g., Table 7. The amount of anti-CD3 antibodies used can also affect the response, as indicated above.
35   Comparing the amounts of cytokines produced, note that the Tr1 cell clones produce a different profile of cytokines from the Th0, Th1, or Th2 clones after anti-CD3 (10 µg/ml) stimulation. With respect to IL-2, these anergic Tr1 cell clones produce less than about 30%, preferably less than about

10%, and typically less than about 2% of the corresponding Th0 or Th1 cell clones. For IL-4, these anergic Tr1 cells produce less than about 30%, preferably less than about 10%, and typically less than about 1% of the corresponding Th0 or Th1
5   cell clones. For IL-5, these anergic Tr1 cell clones produce about 10-20% of the corresponding Th0 cell clones, about 5-10% of the corresponding Th2 cell clones, and about 5-30x the corresponding Th1 cell clones. For IL-10, these anergic Tr1 cells produce about 10-50x as much as corresponding Th0 or Th1
10 cell clones, and about 5-25x as much as corresponding Th2 cells. For IFN-γ, these anergic Tr1 cells produce about 3-20x as much as corresponding Th2 cell clones, and comparable amounts, within a factor of about 2 or 3 of that produced by Th0 or Th1 cell clones. For TNF-α, these Tr1 cell clones
15 produce about 2-5x as much as Th0 or Th2 cell clones, and comparable amounts, within a factor of about 2 or 3, as Th1 cell clones. The anergic Tr1 cell clones also produce only low levels of GM-CSF.

Note also that the duration of IL-10 with antigen can
20 affect the extent of reversibility. While treatments over about 7 days leads to substantial irreversibility with normal amounts of IL-2 or anti-CD3 antibody (10 µg/ml); very high amounts of IL-2, antigen, or anti-CD3 antibody (e.g., 700 µg/ml) will tend to have a greater capacity to either attenuate
25 or reverse the anergy.

IL-10 inhibited in a dose-dependent fashion the alloantigen-induced proliferative responses in primary mixed lymphocyte response. The suppressive effect was optimal when IL-10 was added at the beginning of the cultures suggesting
30 that it acts on the early stages of T cell activation. The proliferative responses were enhanced in the presence of anti-IL-10 mAb, indicating that endogenously produced IL-10 suppresses proliferation in primary MLR. The inhibitory effects of IL-10 were observed irrespective of whether
35 irradiated allogeneic peripheral blood mononuclear cells (PBMC), purified monocytes, or B cells were used as stimulator cells. The reduced proliferative responses were not restored by high concentrations of exogenous IL-2 indicating that the effects of IL-10 are not related to inhibition of IL-2

synthesis. Furthermore, the production of IL-2, IFN-γ, IL-6,
GM-CSF, and TNF-α in primary MLR was diminished by IL-10 and
enhanced in the presence of anti-IL-10 mAb. The strongest
effects were observed on the production of IFN-γ. Although

5   IL-10 reduces the proliferative responses, the ratio of
CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells remained the same in IL-10
treated and control cultures. However, the percentages of
activated CD3$^+$ T cells as judged by CD25$^+$ and HLA-DR$^+$
expression were consistently reduced in the presence of IL-10.

10   Human-IL-10 inhibits the synthesis of IFN-γ and
granulocyte-macrophage colony stimulating factor (GM-CSF)
induced in human PBMC by PHA, anti-CD3 mAb, and IL-2
(Bacchetta, et al. (1989) J. Immunol. 144:902; and Bevan (1984)
Immunol Today 5:128. This inhibition occurs at the

15   transcriptional levels (Altmann, et al. (1989) Nature 338:512;
Bacchetta, et al., supra). Murine IL-10 (m-IL-10) has
pleiotropic activities on different cell types, including
growth promoting effects on thymocytes (Chen, et al. (1991) J.
Immunol. 147:528), cytotoxic T cells (De Koster, et al. (1989)

20   J. Exp. Med. 169:1191), and mast cells (de Waal Malefyt, et al.
(1991) J. Exp. Med. 174:1209). m-IL-10 induces class II MHC
antigen expression on B cells and sustains the viability of
these cells (de Waal Malefyt, et al. (1991) J. Exp. Med.
174:915). Furthermore, IL-10 inhibits cytokine production by

25   macrophages (Bejarano, et al. (1985) Int. J. Cancer 35:327;
Fiorentino, et al. (1989) J. Exp. Med. 170:2081). h- and
m-IL-10 have extensive homology to BCRF-1, an open reading
frame of the Epstein Barr virus (EBV) genome (Azuma, et al.
(1992) J. Exp. Med. 175:353; Bacchetta, et al. (1989) J.

30   Immunol. 144:902). The protein product of BCRF-1, designated
as viral IL-10 (v-IL-10), shares most properties with h-and
m-IL-10 including CSIF activity on human and mouse T cells
(Bacchetta, et al., supra; Bevan, M. J., supra).

         Human-IL-10 and v-IL-10 inhibit antigen specific
35   proliferative responses by reducing the antigen presenting
capacity of human monocytes via downregulation of class II MHC
molecules (Figdor, et al. (1984) J. Immunol. Methods 68:68).
Moreover, IL-10 inhibits cytokine synthesis by LPS or IFN-γ
activated monocytes, including CM-CSF, G-CSF, and the

proinflammatory cytokines IL-1α, IL-1β, IL-6, IL-8, and TNF-α
(Bejarano, et al. (1985) <u>Int. J. Cancer</u> 35:327; Fiorentino, et
al, supra.). Interestingly, LPS activated monocytes produce
high levels of IL-10, and enhanced production of cytokines was

5   observed in the presence of anti-IL-10 mAb indicating an
autoregulatory effect of IL-10 on monokine production
(Bejarano, et al., supra).

Alloreactivity reflects, at least in part, recognition of
foreign MHC molecules plus antigenic peptides of various origin

10 (Fiorentino, et al. (1991) <u>J. Immunol.</u> 146:3444; Fiorentino, et
al. (1991) <u>J. Immunol.</u> 147:3815; Freedman, et al. (1987) <u>J.
Immunol.</u> 139:3260; Go, et al. (1990) <u>J. Exp. Med.</u> 172:1625).
Moreover, alloreactive T cells may recognize conformational
differences between MHC molecules largely independent of the

15 peptides bound, or even on empty MHC molecules (Harding, et al.
(1990) <u>Proc. Natl. Acad. Sci. USA</u> 87:5553; Hsu, et al. (1990)
<u>Science</u> 250:830; Julius, et al. (1973) <u>Eur. J. Immunol.</u> 3:645).
IL-10 inhibits allospecific proliferative responses, and
cytokine production. In addition, the reduced proliferative

20 responses could not be restored by exogenous IL-2.

Thus, the present invention provides means to generate
large quantities of alloantigen specific Tr1 cells by
stimulating host derived CD4+ T cells with donor derived
irradiated PBMC in the presence of IL-10, e.g., for minimally

25 at least about 3 days, preferably at least about 5 days, more
preferably at least about 7 days, and in certain embodiments 9,
11, 13, 15, or more days. These cells can be administered
prior or simultaneously with the transplant (organ or bone
marrow). The transplant event and/or therapy may be with or

30 without administration of IL-10.

The above cell therapy can be extended to treat other
chronic diseases causing by antigens, such as gliadin (e.g.,
gluten) for the treatment of coeliac disease, allergens for the
treatment of chronic allergic diseases (asthma, atopic

35 dermatitis, rhinitis), or GAD (glutamic acid decarboxylase) or
insulin for the treatment of diabetes.

In addition, this may provide treatment for inappropriate
sensitivity to many other potential autoantigens. The cells or
treatment may provide means for induction of long term

tolerance and Tr1 cell development in vivo. Long term, e.g.,
5-15 day treatment with IL-10 may enhance in vivo production of
anergy, with copresentation of appropriate MHC antigens, e.g.,
with class I or class II, or other soluble antigens.

5   The invention also provides means for administration of
IL-10 in order to induce antigen specific Tr1 cells and long
term antigen specific tolerance in vivo for the treatment of
diseases with undesired T-cell activation, e.g., in transplant
rejection, graft versus host disease, parasitic diseases,
10   chronic inflammatory diseases such as Crohn's disease, colitis,
chronic inflammatory eye diseases, chronic inflammatory lung
diseases, and chronic inflammatory liver diseases. See, e.g.,
Frank, et al. (eds.) Samter's Immunologic Diseases Little,
Brown, Boston, MA.

15   In other contexts, it may be useful to administer of IL-10
in order to induce autoantigen specific Tr1 cells and
autoantigen specific tolerance in vivo for the treatment of
autoimmune diseases such as rheumatoid arthritis, diabetes,
multiple sclerosis.

20   In many embodiments, the IL-10 should be typically
administered for a minimum of 5-15 days, preferably at least
about 7 days.

All references cited herein are incorporated herein by
25   reference to the same extent as if each individual reference,
e.g., publication or patent application, was specifically and
individually indicated to be incorporated by reference.

<u>EXAMPLES</u>

30   The following examples serve to illustrate the present
invention. Selection of vectors and hosts as well as the
concentration of reagents, temperatures, and the values of
other variable parameters are only to exemplify applications of
the present invention and the claimed embodiments are not to be
35   limited to the specific embodiments described herein.

Some of the standard methods are described or referenced,
e.g., in Maniatis, et al. (1982) <u>Molecular Cloning, A</u>
<u>Laboratory Manual</u> Cold Spring Harbor Laboratory, Cold Spring
Harbor Press; Sambrook, et al. (1989) <u>Molecular Cloning: A</u>

<u>Laboratory Manual</u> (2d ed.), vols. 1-3, CSH Press, NY; Ausubel,
et al., <u>Biology</u>, Greene Publishing Associates, Brooklyn, NY; or
Ausubel, et al. (1987 and Supplements) <u>Current Protocols in
Molecular Biology</u> Greene/Wiley, New York; Innis, et al. (eds.;
5   1990) <u>PCR Protocols: A Guide to Methods and Applications</u>
Academic Press, N.Y. Methods for protein purification include
such methods as ammonium sulfate precipitation, column
chromatography, electrophoresis, centrifugation,
crystallization, and others. See, e.g., Ausubel, et al. (1987
10   and periodic supplements); Deutscher (1990) "Guide to Protein
Purification" in <u>Methods in Enzymology</u> vol. 182, and other
volumes in this series; and manufacturer's literature on use of
protein purification products, e.g., Pharmacia, Piscataway,
N.J., or Bio-Rad, Richmond, CA. Combination with recombinant
15   techniques allow fusion to appropriate segments, e.g., to a
FLAG sequence or an equivalent which can be fused via a
protease-removable sequence. See, e.g., Hochuli (1989)
<u>Chemische Industrie</u> 12:69-70; Hochuli (1990) "Purification of
Recombinant Proteins with Metal Chelate Absorbent" in Setlow
20   (ed.) <u>Genetic Engineering, Principle and Methods</u> 12:37-98,
Plenum Press, N.Y.; and Crowe, et al. (1992) <u>QIAexpress: The
High Level Expression & Protein Purification System</u> QIAGEN,
Inc., Chatsworth, CA.

Fluorescent activated cell sorting was performed using
25   standard methods on a Becton-Dickinson FACStar PLUS. See,
e.g., Shapiro (1988) <u>Practical Flow Cytometry</u> (2d ed.) Alan
Liss, New York.

Example 1.   <u>Expression of human CSIF in a bacterial host</u>
30   A synthetic human CSIF gene is assembled from a plurality
of chemically synthesized double stranded DNA fragments to form
an expression vector designated TAC-RBS-hCSIF. Cloning and
expression are carried out in a standard bacterial system, for
example, E. coli K-12 strain JM101, JM103, or the like,
35   described by Vieira and Messing (1982) <u>Gene</u> 19:259-268.
Restriction endonuclease digestions and ligase reactions are
performed using standard protocols, e.g., Maniatis, et al.
(1982) <u>Molecular Cloning: A Laboratory Manual</u> Cold Spring
Harbor Laboratory, New York. See also, Ausubel (1987 and

periodic supplements; ed.) <u>Current Protocols in Molecular
Biology</u> Greene/Wiley, NY; Sambrook, et al. (1989) <u>Molecular
Cloning: A Laboratory Manual</u> 2d ed., Cold Spring Harbor
Laboratory, New York.

5   The alkaline method (Maniatis et al., cited above) is used
for small scale plasmid preparations. For large scale
preparations, a modification of the alkaline method is used in
which an equal volume of isopropanol is used to precipitate
nucleic acids from the cleared lysate. Precipitation with cold
10 2.5 M ammonium acetate is used to remove RNA prior to cesium
chloride equilibrium density centrifugation and detection with
ethidium bromide.

           For filter hybridizations Whatman 540 filter circles are
used to lift colonies which are then lysed and fixed by
15 successive treatments with 0.5 M NaOH, 1.5 M NaCl; 1 M Tris HCl
pH 8.0, 1.5 M NaCl (2 min each); and heating at 80° C (30 min).
Hybridizations are in 6 x SSPE, 20% formamide, 0.1% sodium
dodecylsulphate (SDS), 100 µg/ml E. coli tRNA at 42° C for 6
hrs using ^{32}P-labelled (kinased) synthetic DNAs. (20 x SSPE is
20 prepared by dissolving 174 g of NaCl, 27.6 g of $NaH_2PO_49H_2O$,
and 7.4 g of EDTA in 800 ml of H_2O, pH is adjusted to 7.4 with
NaOH, volume is adjusted to 1 liter, and sterilized by
autoclaving). Filters are washed twice (15 min, room
temperature) with 1 x SSPE, 0.1% SDS. After autoradiography
25 (Fuji RX film), positive colonies are located by aligning the
regrown colonies with the blue-stained colonies on the filters.
DNA is sequenced by the dideoxy method, Sanger, et al. (1977)
<u>Proc. Natl. Acad. Sci. USA</u> 74:5463. Templates for the dideoxy
reactions are either single stranded DNAs of relevant regions
30 recloned into M13mp vectors, e.g., Messing, et al. (1981)
<u>Nucleic Acids Res.</u> 9:309, or double-stranded DNA prepared by
the minialkaline method and denatured with 0.2 M NaOH (5 min,
room temperature) and precipitated from 0.2 M NaOH, 1.43 M
ammonium acetate by the addition of 2 volumes of ethanol. DNA
35 is synthesized by phosphoramidite chemistry using Applied
Biosystems 380A synthesizers. Synthesis, deprotection,
cleavage, and purification (7M urea PAGE, elution, DEAE-
cellulose chromatography) are performed, e.g., as described in
the Applied Biosystems 380A synthesizer manual.

Complementary strands of synthetic DNAs to be cloned (400 ng each) are mixed and phosphorylated with polynucleotide kinase in a reaction volume of 50 μl. This DNA is ligated with 1 μg of vector DNA digested with appropriate restriction

5   enzymes, and ligations are in a volume of 50 μl at room temperature for 4 to 12 hours. Conditions for phosphorylation, restriction enzyme digestions, polymerase reactions, and ligation have been described (Maniatis et al., cited above). Colonies are scored for lacZ+ (when desired) by plating on L

10   agar supplemented with ampicillin, isopropyl-1-thio-beta-D-galactoside (IPTG) (0.4 mM) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) (40 mg/ml).

The TAC-RBS vector is constructed by filling-in with DNA polymerase the single BamHI site of the tacP-bearing plasmid

15   pDR540 (Pharmacia). This is then ligated to unphosphorylated synthetic oligonucleotides (Pharmacia) which form a double-stranded fragment encoding a consensus ribosome binding site (RBS) GTAAGGAGGTTTAAC (SEQ ID NO: 5). After ligation, the mixture is phosphorylated and re-ligated with the SstI linker

20   ATGAGCTCAT (SEQ ID NO: 6). This complex was then cleaved with SstI and EcoRI, and the 173 bp fragment isolated via polyacrylamide gel electrophoresis (PAGE) and cloned into EcoRI-SstI restricted pUC19 (Pharmacia) as described below. The sequence of the RBS-ATG-polylinker regions of the final

25   construction (called TAC-RBS) is shown in Fig. 2.

The synthetic IL-10 gene is assembled into a pUC19 plasmid in eight steps. At each step inserts free of deletions and/or inserts can be detected after cloning by maintaining the lacZ(a) gene of pUC19 in frame with the ATG start codon

30   inserted in step 1. Clones containing deletion and/or insertion changes can be eliminated by scoring for blue colonies on L-ampicillin plates containing X-gal and IPTG. Alternatively, at each step sequences of inserts can be readily confirmed using a universal sequencing primer on small scale

35   plasmid DNA preparations, e.g., available from Boehringer Mannheim.

In step 1 the TAC-RBS vector is digested with SstI, treated with T4 DNA polymerase (whose 3' exonuclease activity digests the 3' protruding strands of the SstI cuts to form

blunt end fragments), and after deactivation of T4 DNA
polymerase, treated with EcoRI to form a 173 base pair (bp)
fragment containing the TAC-RBS region and having a blunt end
at the ATG start codon and the EcoRI cut at the opposite end.

5   Finally, the 173 bp TAC-RBS fragment is isolated.

        In step 2 the isolated TAC-RBS fragment of step 1 is mixed
with EcoRI/KpnI digested plasmid pUC19 and synthetic fragment
1A/B which, as shown below, has a blunt end at its upstream
terminus and a staggered end corresponding to a KpnI cut at its

10   downstream terminus. This KpnI end is adjacent to and
downstream of a BstEII site. The fragments are ligated to form
the pUC19 of step 2.

        In step 3 synthetic fragments 2A/B and 3A/B (shown below)
are mixed with BstEII/SmaI digested pUC19 of step 2 (after

15   amplification and purification) and ligated to form pUC19 of
step 3. Note that the downstream terminus of fragment 3A/B
contains extra bases which form the SmaI blunt end. These
extra bases are cleaved in step 4. Also, fragments 2A/B and
3A/B have complementary 9 residue single stranded ends which

20   anneal upon mixture, leaving the upstream BstEII cut of 2A/B
and the downstream blunt end of 3A/B to ligate to the pUC19.

        In step 4 AflII/XbaI digested pUC19 of step 3 (after
amplification and purification) is repurified, mixed with
synthetic fragment 4A/B (shown below), and ligated to form the

25   pUC19 of step 4.

        In step 5 XbaI/SalI digested pUC19 of step 4 (after
amplification and purification) is mixed with synthetic
fragment 5A/B (shown below) and ligated to form the pUC19 of
step 5. Note that the SalI staggered end of fragment 5A/B is

30   eliminated by digestion with HpaI in step 6.

        In step 6 HpaI/PstI digested pUC19 of step 5 (after
amplification and purification) is mixed with synthetic 6A/B
(shown b. ow) and ligated to form the pUC19 of step 6.

        In step 7, ClaI/SphI digested pUC19 of step 6 (after

35   amplification and purification) is mixed with synthetic
fragment 7A/B (shown below) and ligated to form the pUC19 of
step 7.

        In step 8 MluI/HindIII digested pUC19 of step 7 (after
amplification and purification) is mixed with synthetic

fragments 8A/B and 9A/B and ligated to form the final construction. The final construction is inserted into E. coli K-12 strain JM101, e.g., available from the ATCC under accession number 33876, by standard techniques. After culturing, protein is extracted from the JM101 cells and dilutions of the extracts are tested for biological activity.

```
AGCCCAGGCC AGGGCACCCA GTCTGAGAAC AGCTGCACCC ACTTCCCAGG-
   TCGGGTCCGG TCCCGTGGGT CAGACTCTTG TCGACGTGGG TGAAGGGTCC-

tAACCggtac  (SEQ ID NO: 7)
aTTGGc  (SEQ ID NO: 8)
```

                              Fragment 1A/B


```
GtAACCTGCC TAACATGCTT CGAGATCTCC GAGATGCCTT CAGCAGAGTG-
      GACGG ATTGTACGAA GCTCTAGAGG CTCTACGGAA GTCGTCTCAC-

AAGACTTTCTTT  (SEQ ID NO: 9)
TTC  (SEQ ID NO: 10)
```

                              Fragment 2A/B


```
        CAAATGAAGG ATCAGCTGGA CAACTTGTTc TtAAG  (SEQ ID NO: 11)
   TGAAAGAAA GTTTACTTCC TAGTCGACCT GTTGAACAAg AaTTC  (SEQ ID NO: 12)
```

                              Fragment 3A/B


```
GAGTCCTTGC TGGAGGACTT TAAGGGTTAC CTGGGTTGCC AAGCCTTGTC TGAGATGATC-
   CTCAGGAACG ACCTCCTGAA ATTCCCAATG GACCCAACGG TTCGGAACAG ACTCTACTAG-

CAGTTTTAt  (SEQ ID NO: 13)
GTCAAAATaG AtC  (SEQ ID NO: 14)
```

                              Fragment 4A/B


```
CTaGAGGAGG TGATGCCCCA AGCTGAGAAC CAAGACCCAG ACATCAAGGC-
   GAtCTCCTCC ACTACGGGGT TCGACTCTTG GTTCTGGGTC TGTAGTTCCG-

GCATGTtAAC g  (SEQ ID NO: 15)
CGTACAaTTG cagct  (SEQ ID NO: 16)
```

                              Fragment 5A/B


```
AACTCCCTGG GGGAGAACCT GAAGACCCTC AGGCTGAGGC TACGGCGCTG-
   TTGAGGGACC CCCTCTTGGA CTTCTGGGAG TCCGACTCCG ATGCCGCGAC-

TCATCGATct gca  (SEQ ID NO: 17)
AGTAGCTAg  (SEQ ID NO: 18)
```

                              Fragment 6A/B

```
CGATTTCTTC CCTGTCAAAA CAAGAGCAAG GCCGTGGAGC AGGTGAAGAA-
   TAAAGAAG GGACAGTTTT GTTCTCGTTC CGGCACCTCG TCCACTTCTT-
```

```
cGCgTgcatg   (SEQ ID NO: 19)
gCGcAc   (SEQ ID NO: 20)
```

<u>Fragment 7A/B</u>

```
CGCGTTTAAT AATAAGCTCC AAGACAAAGG CATCTACAAA GCCAT-
   AAATTA TTATTCGAGG TTCTGTTTCC GTAGATGTTT CGGTA-
```

```
GAGTGAGTTT GAC   (SEQ ID NO: 21)
CTCA   (SEQ ID NO: 22)
```

<u>Fragment 8A/B</u>

```
ATCTTCATCA ACTACATAGA AGCCTACATG ACAATGAAGA-
CTCAAACTG TAGAAGTAGT TGATGTATCT TCGGATGTAC TGTTA CTTCT-
```

```
TACGAAACTG A   (SEQ ID NO: 23)
ATGCTTTGAC TAcga   (SEQ ID NO: 24)
```

<u>Fragment 9A/B</u>

(Lower case letters indicate that a base differs from that of the
native sequence at the same position.)

Example 2.   <u>Expression of vIL-10 in COS 7 Monkey cells</u>

     A gene encoding the open reading frame for vIL-10 was
amplified by polymerase chain reaction using primers that
allowed later insertion of the amplified fragment into an
EcoRI-digested pcD(SRα) vector (Fig. 1).   The coding strand of
the inserted fragment is shown below (the open reading frame
being given in capital letters).

```
aattcATGGA GCGAAGGTTA GTGGTCACTC TGCAGTGCCT GGTGCTGCTT
TACCTGGCAC CTGAGTGTGG AGGTACAGAC CAATGTGACA ATTTTCCCCA
GACCTAAGAG ATGCCTTCAG TCGTGTTAAA ACCTTTTTCC AGACAAAGGA
CGAGGTAGAT AACCTTTTGC TCAAGGAGTC TCTGCTAGAG GACTTTAAGG
ATGCCAGGCC CTGTCAGAAA TGATCCAATT CTACCTGGAG GAAGTCATGC
CACAGGCTGA AACCAGGACC CTGAAGCCAA AGACCATGTC AATTCTTTGG
GTGAAAATCT AAAGACCCTA CGGCTCCGCC TGCGCAGGTG CCACAGGTTC
CTGCCGTGTG AGAACAAGAG TAAAGCTGTG GAACAGATAA AAAATGCCTT
TAACAAGCTG CAGGAAAAAG GAATTTACAA AGCCATGAGT GAATTTGACA
TTTTTATTAA CTACATAGAA GCATACATGA CAATTAAAGC CAGGTGAg
(SEQ ID NO: 25)
```

Clones carrying the insert in the proper orientation were
identified by expression of vIL-10 and/or the electrophoretic
pattern of restriction digests.   One such vector carrying the
vIL-10 gene was designated pBCRF1(SRα) and was deposited with
the ATCC under accession number 68193 on December 20, 1989.

PBCRF1(SRα) was amplified in E. coli MC1061, isolated by
standard techniques, and used to transfect COS7 monkey cells as
follows: One day prior to transfection, approximately 1.5 x
10^6 COS7 monkey cells were seeded onto individual 100 mm plates

5   in Dulbecco's modified Eagle medium (DME) containing 5% fetal
calf serum (FCS), and 2 mM glutamine. To perform the
transfection, COS7 cells were removed from the dishes by
incubation with trypsin, washed twice in serum-free DME, and
suspended to 10^7 cells/ml in serum-free DME. A 0.75 ml aliquot

10   was mixed with 20 µg DNA and transferred to a sterile 0.4 cm
electroporation cuvette. After 10 minutes, the cells were
pulsed at 200 volts, 96 µF in a BioRad Gene Pulser unit. After
another 10 minutes, the cells were removed from the cuvette and
added to 20 ml of DME containing 5% FCS, 2 mM glutamine,

15   penicillin, streptomycin, and gentamycin. The mixture was
aliquoted to four 100 mm tissue culture dishes. After 12-24
hours at 37° C, 5% CO_2, the medium was replaced with similar
medium containing only 1% FCS and the incubation continued for
an additional 72 hours at 37° C, 5% CO_2, after which the medium

20   was collected and assayed for its ability to inhibit IFN-γ
synthesis.

        10 ml aliquots of freshly isolated PBLs (about 2 x 10^6
cells/ml) were incubated at 37° C with PHA (100 ng/ml) in
medium consisting of (i) 90% DME supplemented with 5% FCS and 2

25   mM glutamine, and (ii) 10% supernatant from COS 7 cells
previously transfected with pBCRF1(SRα). After 24 hours the
cells and supernatants were harvested to assay for the presence
of either IFN-γ mRNA or IFN-γ protein, respectively. Controls
were treated identically, except that the 10% supernatant was

30   from COS 7 cultures previously transfected with a plasmid
carrying an unrelated cDNA insert. The vIL-10-treated samples
exhibited about a 50% inhibition of IFN-γ synthesis relative to
the controls.

35   Example 3.   Expression of vIL-10 in Escherichia coli
        A gene encoding the mature vIL-10 shown in SEQ ID NO: 4
may be expressed in E. coli.
        The cDNA insert of pBCRF1(SRα) is recloned into an M13
plasmid where it is altered twice by site-directed mutagenesis:

first to form a ClaI site at the 5' end of the coding region
for the mature vIL-10 polypeptide, and second to form a BamHI
site at the 3' end of the coding region for the mature vIL-10
polypeptide. The mutated sequence is then readily inserted
5   into the TRPC11 expression vector described below.

The TRPC11 vector was constructed by ligating a synthetic
consensus RBS fragment to ClaI linkers (ATGCAT) and by cloning
the resulting fragments into ClaI restricted pMT11hc (which had
been previously modified to contain the ClaI site). PMT11hc is
10 a small (2.3 kilobase) high copy, AMP^R, TET^S derivative of
pBR322 that bears the πVX plasmid EcoRI-HindIII polylinker
region. (πVX is described by Maniatis, et al. (1982) _Molecular
Cloning: A Laboratory Manual_ Cold Spring Harbor Laboratory)
This was modified to contain the ClaI site by restricting
15 pMT11hc with EcoRI and BamHI, filling in the resulting sticky
ends, and ligating with ClaI linker (CATCGATG), thereby
restoring the EcoRI and BamHI sites and replacing the SmaI site
with a ClaI site. One transformant from the TRPC11
construction had a tandem RBS sequence flanked by ClaI sites.
20 One of the ClaI sites and part of the second copy of the RBS
sequence were removed by digesting this plasmid with PstI,
treating with Bal31 nuclease, restricting with EcoRI, and
treating with T4 DNA polymerase in the presence of all four
deoxynucleotide triphosphates. The resulting 30-40 bp
25 fragments were recovered via PAGE and cloned into SmaI
restricted pUC12. A 248 bp E. coli trpP-bearing EcoRI fragment
derived from pKC101 (described by Nichols, et al. (1983)
Methods in Enzymology 101:155 Academic Press, N.Y.) was then
cloned into the EcoRI site to complete the TRPC11 construction,
30 which is illustrated in Fig. 2. TRPC11 is employed as a vector
for vIL-10 by first digesting it with ClaI and BamHI, purifying
it, and then mixing it in a standard ligation solution with the
ClaI-BamHI fragment of the M13 containing the nucleotide
sequence coding for the mature BCRF1. The insert-containing
35 TRPC11, referred to as TRPC11-BCRF1, is propagated in E. coli
K12 strain JM101, e.g., available from the ATCC under accession
number 33876.

Example 4.   <u>IL-10 effects on generation of alloreactivity in</u>
<u>primary MLR</u>

Cells were cultured in Yssel's medium (Koulova, et al.
5   (1991) <u>J. Exp. Med.</u> 173:759) supplemented with 10% pooled heat
inactivated human AB serum.

The neutralizing anti-IL-10 mAb 19F1 was raised against
v-IL-10 and efficiently neutralized h- and v-IL-10 (Bejarano,
et al. (1985) <u>Int. J. Cancer</u> 35:327).   The BB10 mAb which
10   recognizes the IL-2R p55 chain was a kind gift of Dr. J.
Wijdenes (CRTS, Bensançon, France).   Murine anti-CD3 (anti-
Leu-4, IgG1), anti-CD4 (Anti-Leu-3a, IgG1), anti-CD8 (anti-Leu-
2a, IgG2a), anti-CD14 (anti-Leu-M3, IgG2b), anti-CD19 (anti-
Leu-12, IgG1), anti-CD25 (anti-IL2R p55, IgG1), anti-CD56
15   (anti-Leu-19, IgG1), anti-HLA-DR (clone L243, IgG2a) mAb and
control mAb of appropriate isotypes were obtained from Becton
Dickinson (Mountain View, CA).

Buffy coat preparations were obtained from the Blood Bank
of Stanford University Hospital.   PBMC were isolated by density
20   gradient centrifugation over Ficoll-hypaque (Pharmacia,
Uppsala, Sweden).

For purification of T cells, PBMC were depleted of
monocytes by plastic adherence and iron phagocytosis using the
method of Linsley, et al. (1991) <u>J. Exp. Med.</u> 173:721.   Non-
25   adherent cells were passed through nylon wool as described in
MacNeil, et al. (1990) <u>J. Immunol.</u> 145:4167).   Thereafter, NK
cells were removed by depletion with magnetic beads.   Briefly,
following staining with saturating concentrations of anti-CD56
mAb for 30 min at 4° C, cells were washed 2 times with Hank's
30   balanced salt solution (HBSS), and subsequently rosetted with
magnetic beads coated with sheep anti-mouse IgG (Dynabeads M-
450 sheep anti-mouse IgG, Dynal AS, Oslo, Norway) at a bead to
cell ratio of 40:1.   The mixture was incubated for 30 min at 4°
C with gentle shaking before removal of rosetted cells with the
35   magnetic particle concentrator according to the manufacturer's
recommendations.   The resulting cell preparations were >99%
CD3+, <1% CD14+, <1% CD19+, <1% CD56+.

For isolation of CD14+ monocytes, PBMC were stained with
PE-conjugated CD14 mAb (Becton-Dickinson, Mountain View, CA),
40   washed twice in HBSS and thereafter sorted in CD14+ and CD14-

populations using a FACStar-Plus (Becton-Dickinson, Sunnyvale, CA). Reanalysis of the sorted populations showed that more than 99.5% of the purified cells were CD14+. In some experiments monocytes were isolated from peripheral blood by
5   density centrifugation in a blood component separator, followed by centrifugal elutriation as described in detail by Moore, et al. (1990) Science 248:1230. These monocyte preparations were 95% pure, as judged by nonspecific esterase staining.

Purified B lymphocytes were obtained by magnetic bead
10   depletion. Non-adherent PBMC were incubated with saturating concentrations of anti-CD3, CD4, CD8, CD14, and CD56 mAb for 30 min at 4° C. The cells were washed twice in HBSS and thereafter rosetted with magnetic beads coated with sheep anti-mouse IgG (Dynal, AS, Oslo, Norway) at a 40:1 bead to cell
15   ratio. Subsequently, the rosetted cells were depleted as described above. The resulting population consisted of >98% CD19+ cells.

For proliferation assays, PBMC or highly purified T cells (1 x 10^5 cells/well), were stimulated by various irradiated
20   (4000 rad) allogeneic stimulator cells. PBMC, CD14+ monocytes, monocytes separated by centrifugal elutriation, or purified B lymphocytes were used as stimulator cells at R:S ratios of 1:1, 5:1, 5:1, and 3:1, respectively. Cultures were carried out in triplicate in 96-well flat-bottomed microtiter plates in the
25   absence or in the presence of IL-10 in 200 μl medium.

Cultures were pulsed with [3H]TdR during the last 10 hr of a 5 day incubation period and harvested onto fiberglass filters and the radioactivity determined by liquid scintillation counting. The results are expressed as cpm of [3H]TdR
30   incorporation and represent the means of multiple cultures.

For bulk cultures, PBMC or highly purified T cells were cultured with irradiated allogeneic cells at the R:S ratios described above in 50 ml flasks at a concentration of 1 x 10^6 responder cells/ml in the presence or in the absence of 100
35   U/ml of IL-10. Five to six days later the supernatants were collected and frozen at -20° C for determination of their cytokine contents, whereas the cells were recovered for phenotype analysis.

For fluorescence analysis, cells (10^5) recovered from the bulk cultures were incubated in V-bottomed microtiter plates (Flow Laboratories, city, state) with 10 μl of purified PE-conjugated mAb for 30 min at 4° C. In the double-labeling experiments, the cells were washed twice in 1% normal mouse serum after the FITC labeling, and a PE-conjugated mAb was added. The cells were washed twice with HBSS containing 1% BSA and 0.02M NaN_3 and thereafter analyzed on a FACScan.

For cytokine determinations, supernatants collected from bulk cultures at day 5 or 6 were assayed for the content of GM-CSF IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-6 by lymphokine specific ELISA as described in Öhlen, et al (1990) <u>J. Immunol.</u> 145:52. For the quantification of IL-2 production, cultures were carried out in the presence of 10 μg/ml of the anti-IL-2 receptor antibody, BB10, in order to minimize IL-2 consumption. Supernatants were harvested after 72 hr and the IL-2 levels were determined by specific ELISA. The sensitivity of the various ELISA were: 40 pg/ml for IL-4; 20 pg/ml for IL-2, IL-5 and IL-6; 50 pg/ml for GM-CSF; and 100 pg/ml for TNF-α, and IFN-γ.

To determine the effects of IL-10 on the proliferative responses in classical one-way primary MLR. PBMC were stimulated with irradiated allogeneic PBMC in the absence or presence of different concentrations of IL-10. In Fig. 3 it is shown that IL-10 inhibited the proliferative responses in a dose-dependent fashion.

As shown in Fig. 3, PBMC (1 x 10^5/well) and allogeneic irradiated PBMC (1x10^5/well) (PBMC donor A x irradiated PBMC donor B (A): PBMC donor B x irradiated PBMC donor A (B)) were cultured for 5 days in the presence of increasing concentrations of IL-10 (open bars) and anti-IL-10 mAb (solid bars). MLR were carried out in the absence (solid bars) or in the presence (hatched bars) of 100 U/ml IL-10 and increasing concentrations of anti-IL-10 mAb.

Significant inhibitory effects on proliferation were already observed at IL-10 concentrations as low as 1 U/ml, whereas maximal inhibitory effects (ranging from 33 to 95% inhibition in different experiments) were obtained at IL-10 concentrations of 100 U/ml. These inhibitory effects of IL-10

on PBMC proliferation were completely neutralized by the
anti-IL-10 mAb indicating the specificity of the inhibition
(Fig. 3C). The proliferative responses in MLR carried out in
the presence of the neutralizing anti-IL-10 mAb were

5   significantly enhanced, indicating that endogenously produced
IL-10 is responsible for suppressing proliferative responses in
primary MLC\R.

Recently it was demonstrated that IL-10 strongly reduces
the antigen (Ag) presenting (AP) capacity of monocytes through

10   downregulation of class II MHC antigens. In contrast, class II
MHC expression and AP-capacity of EBV-transformed B cells
(EBV-LCL) were not affected by IL-10 (Figdor, et al. (1984) $\underline{J.}$
$\underline{Immunol. Methods}$ 68:68). Therefore, whether the inhibitory
effects of IL-10 in MLR were observed only when monocytes were

15   present was investigated. For this purpose highly enriched
T cells obtained by negative selection were used as responder
cells. Purified monocyte populations enriched either by
centrifugal elutriation or by direct sorting of CD14$^+$ cells
from PBMC, purified B lymphocytes, and EBV-LCL were used as

20   stimulator cells.

Fig. 4A-4D show the effects of IL-10 on the proliferative
responses of purified T cells stimulated with various
allogeneic cells. Purified T cells (1 x 10^5/well) were
cultured for 5 days with allogeneic irradiated elutriated

25   monocytes (2 x 10^4/well) (Fig. 4A), positively sorted CD14$^+$
monocytes (2 x 10^4/well) (Fig. 4B), purified B cells (3.3 x
10^4/well) (Fig. 4C), EVC-LCL (1 x 10^4/well) (Fig. 4D) in the
presence of increasing concentrations of IL-10.

As can be seen in Fig. 4A-4D, IL-10 strongly inhibited the

30   proliferative responses induced by allogeneic monocytes
independently of whether the monocytes were obtained by
centrifugal elutriation (Fig. 4A) or positively sorted by the
FACS (Fig. 4B), whereas the proliferative responses towards
allogeneic EBV-LCL remained unaffected (Fig. 4D). As observed

35   with specific proliferative responses to soluble antigens,
these results indicate that allospecific proliferation is
blocked when allogeneic monocytes, but not when allogeneic
EBV-LCL are used as stimulator cells. Interestingly, the
proliferative responses induced by freshly isolated highly

purified allogeneic B cells were also inhibited by IL-10 (Fig. 4C), indicating that the suppressive effect of IL-10 is also present when B cells are used as stimulators, despite the fact that IL-10 has no measurable effect on class I or class II MHC

5   expression on these cells.

Kinetic experiments revealed that the effect of IL-10 on MLR-induced proliferation decreased gradually with time. These results are shown in Fig. 5, wherein PBMC (1×10^5/well) and allogeneic irradiated PBMC (1×10^5/well) were cultured for 5

10   days, and increasing concentrations of IL-10 were added at times indicated. IL-10 was most effective when added at the beginning of the primary cultures: if added at days 2 or 3 after the onset of the cultures, the effects were only marginal and no clear dose response effects were observed. These

15   results indicate that IL-10 acts on the early stages of activation of T cells in MLR.

Example 5:   IL-10 effects on cytokine production in MLC

IL-10 has been shown to reduce IFN-γ and GM-CSF production

20   by PBMC activated by anti-CD3 or PHA (Bacchetta, et al. (1989) J. Immunol. 144:902; Bevan (1984) supra). In addition, IL-10 inhibits the production of cytokines by monocytes (Bejarano, et al. (1985) Int. J. Cancer 35:327; Fiorentino, et al., supra.). To determine the effect of IL-10 on cytokine production in one-

25   way MLR, allogeneic PBMC were used as responder and as stimulator cells. Human PBMC were cultured for 5 days alone or with allogeneic irradiated PBMC in the absence and in the presence of IL-10 or the anti-IL-10 mAb, 19F1. Supernatants were collected at day 5 and assayed for their cytokine content.

30   Production of cytokines was determined in the supernatants by cytokine-specific ELISAs. Table 1 shows the effect of exogenous and endogenous IL-10 on cytokine production by alloantigen-stimulated lymphocytes.

Table 1

Condition		Cytokine				
IL-10 (U/ml)	αIL-10 (µg/ml)	IL-6 (ng/ml)	IL-10 (ng/ml)	GM-CSF (pg/ml)	TNF-α (pg/ml)	IFN-γ (ng/ml)
A 0		22.2	572	109	79	<1
1		20.5		41	61	<1
10		13.0		11	38	<1
100		12.1		16	47	<1
	.05	22.9		144	144	<1
	.5	25.8		165	111	<1
	5	24.9		172	51	<1
A+B 0		35.4	1473	1744	141	29.5
1		35.3		928	151	20.8
10		23.9		784	82	18.1
100		24.7		612	66	11.4
	.05	36.9		2372	137	33.1
	.5	39.1		2355	137	45.3
	5	39.1		3487	250	43.8

In Table 1 it is shown that IFN-γ, IL-6, GM-CSF, and TNF-α
were produced in MLR, and that IL-10 inhibited the production

5   of these cytokines to various extents.

No significant IL-4 production was detected and the levels
of IL-5 were below 100 pg/ml. The production of IL-10 ranged
from 1000 to 3000 pg/ml in different experiments. The
strongest inhibitory effects of exogenous IL-10 were observed

10   on the production of IFN-γ, whereas the weakest inhibitory
effects were observed on IL-6 production.

Increased IFN-γ, GM-CSF, and TNF-α levels were observed in
supernatants of MLR carried out in the presence of anti-IL-10
mAb, as shown in Table 1. These enhancing effects of anti-

15   IL-10 mAb on cytokine production were dose-dependent. Taken
together these results indicate that both endogenous and
exogenous IL-10 reduce the production of the cytokines tested.
To evaluate the effect of IL-10 on IL-2 production in MLR, and
to minimize IL-2 consumption by activated T cells, the cultures

20   were carried out in the presence or in the absence of IL-10 and
the anti-IL-2 receptor mAb BB10. In these experiments IL-10
prevented IL-2 production in a dose-dependent manner (Fig. 6).
No measurable levels of IL-2 could be detected when IL-10 was
added at 100 U/ml. In Fig. 6, PBMC (1 x 10^5/well) and

25   allogeneic irradiated PBMC (1 x 10^5/well) were cultured with
increasing concentrations of IL-10 and in the presence or in

the absence of 10 µg/ml of the anti-IL-2 R antibody, BB10.
Three days later the supernatants were harvested and assayed
for the IL-2 content by cytokine specific ELISA.

To investigate whether the inhibitory effects of IL-10
5   were observed in the presence of exogenous IL-2, MLR were
carried out with various concentrations of IL-2. In Figs. 7A-
7B, the effects of exogenous IL-2 on the reduced alloantigen-
induced proliferative response of T cells induced by IL-10 is
shown. Purified T cells (10^5/well) stimulated with allogeneic
10   irradiated PBMC (10^5/well) (Fig. 7A), or purified B cells (3.3
x 10^4/well) (Fig. 7B), were cultured with increasing amounts of
IL-2 in the absence (open symbols) or in the presence (closed
symbols) of 100 U/ml of IL-10. These show that addition of
increasing amounts of IL-2 to MLR, in which purified T cells
15   were used as responders and PBMC or purified B cells as
stimulators, enhanced the proliferation both in the absence or
in the presence of IL-10. However, the inhibitory effects of
IL-10 were still present when IL-2 was added at concentrations
up to 100 U/ml [10 U/ml are sufficient to saturate high
20   affinity IL-2 receptor (IL-2R)]. Similarly, addition of 400
U/ml of IL-4, which has T cell growth factor activity (Panina-
Bordignon, et al. (1991) _Science_ 252:1548), failed to restore
the reduced proliferative responses induced by IL-10. Taken
together, these results demonstrate that the lack of IL-2 is
25   not the limiting factor responsible for the reduced
proliferative responses observed when MLR are carried out in
the presence of IL-10.

Example 6.   IL-10 effects on propagation of activated T cells
30   in MLC

To determine whether the reduced proliferative responses
in MLR in the presence of IL-10 differentially affected CD4^{+} or
CD8^{+} T cell subsets, purified T cells were cultured with
allogeneic irradiated PBMC, purified B cells, or monocytes in
35   the absence or presence of IL-10 (100 U/ml). Six days later
the recovered T cells were counted and phenotype-determined by
indirect immunofluorescence. The proportions of CD3^{+}CD4^{+} and
CD3^{+}CD8^{+} cells were determined. Table 2 shows the effect of
IL-10 on alloantigen-stimulated T cells.

Table 2

	T cell count x 10^{-6}/ml		% positive cells							
			CD3$^+$CD4$^+$		CD3$^+$CD8$^+$		CD3$^+$CD25$^+$		CD3$^+$DR$^+$	
	---	IL-10	---	IL-10	---	IL-10	---	IL-10	---	IL-10
experiment 1										
T + PBMC	1.3	.76	69	73	21	24	4	2	3	2
T + B cells	1.2	.72	55	62	28	28	24	18	19	8
experiment 2										
T + PBMC	1.4	1.0	ND^b	ND	ND	ND	39	27	36	32
T + B cells	.90	.68	ND	ND	ND	ND	20	8	18	4
T + monocytes^a	1.2	.40	ND	ND	ND	ND	52	24	43	25
experiment 3										
T + PBMC	1.02	.37	77	78	18	16	16	13	15	9
T + monocytes^c	.50	.17	76	79	19	16	7	4	8	5

^{a} Negatively sorted monocytes
^{b} ND = not done
^{c} positively sorted monocytes (CD14$^+$)

In Table 2, it is shown that the total T cell number decreased by 30 to 60% when the T cells were stimulated with allogeneic PBMC, purified monocytes, or B cells in the presence of IL-10. However, the proportion of CD4$^+$ and CD8$^+$ T cells remained the same, indicating that IL-10 has no preferential effect on each of these T cell subsets.

In contrast, the proportion of activated T cells expressing CD25 and HLA-DR antigens was consistently reduced in IL-10 containing cultures. The strongest reduction was usually observed in MLR where purified CD3$^+$ T cells were stimulated with purified B cells or monocytes.

Thus, IL-10 reduces in a dose-dependent fashion the proliferation of alloresponsive T cells in classical one-way primary MLR in which allogeneic PBMC of two different donors were used as responder and irradiated stimulator cells, respectively. These inhibitory effects were completely neutralized by an anti-IL-10 mAb, demonstrating the specificity of the inhibition. The proliferative responses were considerably enhanced in the presence of the anti-IL-10 mAb, indicating that endogenous IL-10 production is responsible for suppression of proliferative responses in MLR.

IL-10 also reduced the proliferative responses in MLR where highly purified T cells were used as responders and

purified monocytes as stimulators. Interestingly, IL-10 was
ineffective when purified T cells were stimulated by irradiated
allogeneic EBV-LCL. IL-10 strongly blocked the specific
proliferative responses of T cells or T cell clones towards

5 soluble antigens or antigenic peptides when monocytes, but not
when EBV-LCL, were used as APC. This reduced antigen
presenting capacity was found to be associated with the
downregulatory effect of IL-10 on class II MHC expression on
monocytes. In contrast, IL-10 did not affect class II MHC

10 expression on EBV-LCL (Figdor, et al. (1984) $\underline{J. Immunol.}$
$\underline{Methods}$ 68:68). From these data it was concluded that the
reduced antigen specific proliferative T cell responses
reflected prevention of activation of the responder cells,
rather than a direct suppressive effect on T cell

15 proliferation. This conclusion was further supported by the
reduced Ca^{2+} fluxes in the responder T cell clones activated in
the presence of IL-10 (Figdor, et al (1984) $\underline{J. Immunol. Methods}$
68:68).

Interestingly, MLR-induced proliferation was not only

20 inhibited by IL-10 when monocytes, but also when purified B
cells were used as stimulators. Several studies have shown
that T cell recognition of MHC alloantigens is mechanistically
similar to recognition of viral, bacterial or other foreign
protein antigens (Fiorentino, et al. (1991) $\underline{J. Immunol.}$

25 146:3444; Fiorentino, et al. (1991) $\underline{J. Immunol.}$ 147:3815;
Freedman, et al. (1987) $\underline{J. Immunol.}$ 139:3260; Go, et al. (1990)
$\underline{J. Exp. Med.}$ 172:1625). Recently, it has been shown that a
significant proportion of MHC class II alloreactive T cell
clones recognize processed determinants from human serum

30 proteins in association with allogeneic class II molecules
(Fiorentino, et al. (1991) $\underline{J. Immunol.}$ 147:3815). In contrast
to the situation where new MHC-peptide complexes have to be
formed to activate antigen-specific T cell clones (Rötzchke, et
al. (1991) $\underline{J. Exp. Med.}$ 174:1059), there is no evidence to

35 indicate that new allo-MHC-peptide complexes must be formed on
monocytes and B cells to stimulate T cells in a MLR. It is
unlikely that the inhibitory effects of IL-10 on MLR-induced
T cell proliferation can be solely attributed to a
downregulation of MHC class II expression on the monocytes.

It has been demonstrated that, in addition to crosslinking of the TCR/CD3 complex by specific alloantigen, LFA-1 - ICAM-1 interactions are required for cytokine production by allospecific T cells (Santos-Aguado, et al. (1989) Proc. Natl.

5   Acad. Sci. USA 86:8936). Furthermore, CD28 - B7/BB1 interactions have been shown to be necessary for induction of alloantigen-specific activation of resting T cells, resulting in cytokine production, proliferation, and cytotoxic activity (Spits, et al. (1987) J. Immunol. 139:1143; Thompson-Snipes, et

10 al. (1991) J. Exp. Med. 173:507; Vieira, et al. (1991) Proc. Natl. Acad. Sci. USA 88:1172). B7 is weakly expressed on resting B cells and monocytes, but is elevated following activation of these cells. Hokochi, et al. (1982) J. Immunol. 128:823; Yssel, et al. (1986) Eur. J. Immunol. 16:1187).

15 However, it could be ruled out that the reduced proliferative and cytotoxic alloresponses were due to downregulatory effects of IL-10 on the expression of either TCR/CD3 or these accessory molecules.

The reduced proliferative responses towards alloantigens

20 also reflects prevention of activation of the responder T cells. IL-10 had to be present from the onset of the cultures to exert its maximal inhibitory effects. In addition, the proportion of activated T cells, as judged by the expression of CD25 and HLA-DR antigens, was considerably lower

25 in the IL-10 containing cultures as compared to the control MLR. Although the total number of CD3$^+$ T cells generated in MLR carried out in the presence of IL-10 was reduced, IL-10 did not preferentially affect the responses of CD4$^+$ or CD8$^+$ T cells, since the proportions of these T cells subsets were

30 comparable to those in control MLR carried out in the absence of IL-10. The reduced expression of CD25 indicates that the inhibitory effect on T cell proliferation in a MLR is not a mere consequence of the cytokine inhibitory activity of IL-10. This is supported by the finding that IL-10 also reduces the

35 proliferative responses when exogenous IL-2 is added at concentrations that are sufficient to saturate high affinity IL-2 receptors. Collectively, these data suggest that IL-10 reduces the stimulatory capacity of PBMC, monocytes, and normal B cells in MLR.

43

The levels of cytokines produced in MLR in which total
allogeneic PBMC were used as responder and stimulator were also
significantly reduced in the presence of exogenous IL-10. The
amounts of IL-2, IFN-γ, TNF-α, and GM-CSF were approximately
5   two to three fold lower than those of control MLR carried out
in the absence of IL-10. IL-6 production was much less
affected, which may be due to the fact that monocytes present
in these cultures already produced considerable amounts of this
cytokine very early after activation, before the suppressor
10   activity IL-10 becomes effective (Bejarano, et al. (1985) _Int._
J. Cancer 35:327).

Example 7.   _IL-10 and tolerance in SCID Patients_
The T cell repertoire and mechanism of tolerance in two
15   patients with severe combined immunodeficiency (SCID)
transplanted with HLA mismatched fetal liver stem cells was
investigated. Two very young SCID children were transplanted
17 and 5 years ago with fetal liver stem cells from fully HLA
disparate donors. Patient SP received 2 fetal liver stem cell
20   transplantations and in both cases syngeneic fetal thymus was
simultaneously injected. Although standard HLA typing showed
engraftment of cells only from the second donor, a more precise
cytofluorometric analysis, using monoclonal antibodies specific
for polymorphic HLA determinants, indicated that 10-20% of the
25   T lymphocytes were actually from the first donor. The second
patient, RV, received 7 fetal liver stem cell transplantations,
but only one donor cell population could be identified in the
peripheral blood (Table 3).

Table 3
HLA TYPING

	A	C	B	DR	DQ
_SP_					
recipient	3-33	6	14-47	4-5	3
1st donor	2-11	4	27-62	1-8	1
2nd donor	1-2	0	8-18	3-9	3
_RV_					
recipient	2-31	4-7	-62	8-10	4-5
donor	2-30	4	8-35	11-13	6-7

30

In such patients, sustained engraftment of donor T cells
was observed after transplantation, whereas B cells and
monocytes were of host origin (Table 4).

Table 4
CHIMERISM

$\alpha\beta TCR^+$ T cells	
	Donor Origin
$\gamma\delta TCR^+$ T cells	
Monocytes	
	Host Origin
B cells	
	Host Origin -> Patient SP
NK cells	
	Donor Origin -> Patient RV

5

Despite this state of split chimerism within cells of the
immune system, complete reconstitution was achieved and normal
in vivo and in vitro antibody responses to recall antigens were
10   observed. This is due to the ability of donor T cells to
cooperate with the APC of the host, across the allogeneic
barrier. In particular, these studies demonstrated that
tetanus toxoid (TT) specific T cell clones of donor origin,
isolated from the peripheral blood of patient SP, can recognize
15   the antigen (Ag) processed and presented by host B cells, EBV
transformed B cell lines, and NK cell clones. In contrast,
none of the Ag specific T cell clones tested recognized TT
presented by the class II HLA antigen expressed by the donor
cells, Roncarolo, et al. (1989) _J. Exp. Med._ 167:1523.
20   The chimerism in the NK population differed in the two
patients, as shown in Table 4. In one case, fresh NK cells and
NK cell clones showed the HLA phenotype of the host; in the
other case, they were of donor origin. These NK cells
expressed the CD16 and CD56 antigens and displayed normal
25   cytotoxic activity against a variety of NK sensitive targets.
These findings suggest that the presence of host or donor
functional NK cells do not prevent stable engraftment of donor
T cells after fetal stem cell transplantation. Despite the
coexistence of lymphoid cells with major and/or minor
30   histocompatibility antigen differences, complete tolerance was

45

achieved in vivo in these two patients and no signs of acute or
chronic graft versus host disease were observed. Furthermore,
in vitro studies showed that specific nonresponsiveness by the
donor T cells towards the HLA antigens expressed by the host
5   was present in a primary mixed leucocyte culture (MLC), whereas
the proliferative responses against allogeneic cells were
normal. At the clonal level, however, the findings have
differed. Host-reactive proliferative and cytotoxic T cell
clones of donor origin recognizing either HLA class I or HLA
10   class II antigens of the host have been derived from the
peripheral blood of both patients. In contrast to what has
been reported in SCID patients transplanted with marrow from
HLA-haploidentical parental donors (Keeren, et al. Hu. Immunol.
29:42, (1990)), no donor-reactive T cell clones could be
15   isolated in these two patients. Furthermore, in patient RV, no
T cell clones specific for the HLA class I locus A antigens of
the host that were shared by the donor could be identified.
Frequency analysis using a modified limiting dilution assay
confirmed the lack of donor reactivity and demonstrated that
20   the frequency of CD8$^+$ host-reactive T cells was in the same
range as the frequency of T cells reacting against third party
HLA antigens. Thus, host-reactive cells are not clonally
deleted from the donor T cell repertoire. Presumably, such
host-reactive T cells are under regulation since clinical
25   manifestations of graft versus host disease were not evident in
these patients. One possibility is that the host-reactive
cells are anergic in vivo and that in vitro stimulation in the
presence of IL-2 can break this anergy. It is known that host-
reactive T cells display a peculiar pattern of lymphokine
30   production after polyclonal and antigen-specific stimulation.
None of the CD4$^+$ as well as the CD8$^+$ T cell clones are able to
secrete IL-4 whereas they synthesize normal levels of IL-2,
IL-5, and GM-CSF after polyclonal activation. IFN-γ production
by these clones is usually very high. In addition, IL-10
35   production by CD4$^+$ host-reactive T-cell clones of patent RV is
extremely high after antigen-specific stimulation and seems
inversely correlated to the low IL-2 synthesis. Furthermore,
addition of exogenous IL-10 can significantly suppress the
proliferative responses of CD4$^+$ host-reactive T cell clones in

vitro. Therefore, IL-10 production by host-reactive T cells
may play an important role in down-regulating their responses
in vivo..

5   The patients are now (1992) age 17 and 5 years old,
healthy, and show normal immunoresponses to recall antigens.
Their T cells are of donor origin, whereas monocytes and B
cells remained of the host. The NK cells have different
sources since in one patient they derive from the donor and in
the other one from the host. Despite the HLA mismatch between
10   donor and host cells, no acute or chronic graft versus host
disease was observed. In vitro experiments with PBMC showed
specific nonresponsiveness for the HLA antigens expressed by
the host cells. However, an extensive clonal analysis showed
that CD4+ and CD8+ host-reactive T cell clones recognizing
15   class II and class I HLA molecules of the host, respectively,
were present in the peripheral blood of both patients.
Limiting dilution experiments indicated that the frequency of
CD8+ host-reactive cells was in the same range as that observed
for alloreactive T cells. In contrast, no donor reactive CD8+
20   T cells could be isolated. Host-reactive CD4+ and CD8+ T cell
clones were normal in their capacity to produce IL-2, IFN-γ,
GM-CSF, and IL-5, but they failed to synthesize IL-4. In
addition, CD4+ T cell clones from patient RV secreted very high
levels of IL-10. Exogenous IL-10 was able to inhibit the
25   proliferative responses of the CD4+ host-reactive T cell
clones. The host-reactive cells are not deleted from the donor
T cell repertoire following allogeneic fetal liver stem cell
transplantation. Therefore, in vivo tolerance between the host
and the donor is maintained by a peripheral autoregulatory
30   mechanism in which cytokines may play a role.

Example 8. Induction of Long Term Antigen-Specific Anergy in
            human CD4+ T cells

       Human CD4+ T cells, activated by allogeneic monocytes in a
35   primary MLR in the presence of exogenous IL-10, specifically
failed to proliferate after restimulation with the same
alloantigens. A comparable state of T-cell unresponsiveness
could be induced by activation of CD4+ T cells by crosslinked
anti-CD3 mAbs in the presence of exogenous IL-10. The anergic
40   T cells failed, upon restimulation with alloantigen or anti-CD3

mAb, to produce IL-2, IL-5, IL-10, IFN-γ, TNF-α, and GM-CSF.
However, restimulation of anergized T cells with anti-CD3 mAbs
induced normal Ca^{2+} fluxes and resulted in increased CD3, CD28,
and class II MHC expression indicating that calcineurin
5   mediated signaling occurs in these anergic cells. The IL-10-
induced anergic state was long-lasting. T-cell anergy could
not be reversed following restimulation of the cells with anti-
CD3 mAbs and anti-CD28 mAbs, although CD3 and CD28 expression
was normal. However, the expression of the IL-2R α chain was
10   not upregulated, which may account for the failure of exogenous
IL-2 to reverse the anergic state. Interestingly, anergic T
cells and their non anergic counterparts showed comparable
levels of proliferation and cytokine production following
activation with PMA and Ca^{2+} ionophore indicating that a direct
15   activation of a PKC dependent-pathway can overcome the
tolerizing effect of IL-10. Taken together, these data
demonstrate that IL-10 induces T-cell anergy and therefore may
play an important role in the induction and maintenance of
antigen-specific T-cell tolerance.
20   IL-10 has been shown to inhibit antigen-specific
activation and proliferation of human peripheral blood T cells
and T cell clones belonging to the Th0, Th1, or Th2 subsets.
Yssel, et al. (1992) J. Immunol. 149:2378-2384; and de Waal
Malefyt, et al. (1991) J. Exp. Med. 174:915-924. These
25   inhibitory effects were indirect and mediated through
inhibition of the function of antigen-presenting cells (APCs).
Macatonia, et al. (1993) J. Immunol. 150:3755-3765; Caux, et
al. (1994) Int. Immunol. 6:1177-1185; Pecanha et al. (1993) J.
Immunol. 150:3215-3223; and Ding and Shevach (1992) J. Immunol.
30   148:3133-3139. IL-10 regulates constitutive and IFN-γ or IL-4
induced class II MHC expression on monocytes, dendritic cells,
and Langerhans cells. de Waal Malefyt, et al. (1991) J. Exp.
Med. 174:915-924; and Peguet-Navarro, et al. (1994) Eur J.
Immunol. 24:884-891. In addition, IL-10 inhibits the
35   expression of CD54 (ICAM-1, the ligand for LFA-1), and of CD80
and CD86 (ligands for CD28) which function as important
costimulatory molecules for T cell activation. Ding, et al.
(1993) J. Immunol. 15:1224-234; Willems, et al. (1994) Eur. J.
Immunol. 24:1007-1009; Chang, et al. (1995) Eur. J. Immunol.

25:394-398. More recently, it has been shown that IL-10 also has a direct effect on CD4^{+} T by suppressing IL-2 secretion. de Waal Malefyt, et al. (1993) J. Immunol. 150:4754-4765; and Taga, et al. (1993) Blood 81:2964-2971.

5   Similar to its inhibitory effects on T cell proliferation in response to soluble antigens, IL-10 strongly reduced the proliferation of human alloreactive cells in mixed lymphocyte reactions (MLR) and the levels of cytokines produce in these MLR were significantly reduced in the presence of exogenous IL-

10 10. Bejarano, et al. (1992) Int. Immunol. 4:1389-1397 and above. In addition, IL-10 suppressed the proliferative responses of CD4^{+} allogeneic T cell clones. In parallel with the reduced proliferation, reductions in the levels of IL-2, IL-5, GM-CSF, and IFN-γ production by these T cell clones were

15 observed. Roncarolo (1995) "Interleukin-10 and transplantation tolerance" in de Waal Malefyt and de Vries (eds.) Interleukin-10 Landes Company, Austin, Texas, p. 113-120.

      Recently, it was reported that in SCID patients successfully transplanted with HLA-mismatched hematopoietic

20 cells, CD4^{+} T cell clones specifically recognizing the host alloantigens produce very low levels of IL-2 after antigenic stimulation, but secrete high amounts of IL-10, which partially inhibits their proliferation in vitro. Bacchetta, et al. (1994) J. Exp. Med. 179:493-502. Furthermore, PBMC from these

25 SCID patients express considerably higher levels of IL-10 transcript compared with PBMC of normal controls, especially in the non-T cell subset. Bacchetta, et al. (1994) J. Exp. Med. 179:493-502. These results suggest that the high expression of IL-10 detected in SCID human chimeras may play a key role in

30 the maintenance of in vivo tolerance, by inducing an anergic state in donor-derived T cells specific for the host alloantigens.

      Optimal activation and expansion of alloreactive T cells requires, in addition to ligation of the TCR complex,

35 costimulatory signals provided by one or more accessory molecules expressed on alloantigen-presenting cells. Engagement of the TCR by antigens without costimulation results in T cell anergy. De Silva, et al. (1991) J. Immunol. 147:3261-3267; Jenkins, et al. (1990) J. Immunol. 144:16-22;

Schwartz (1990) <u>Science</u> 248:1349-1356; and Sloan-Lancaster, et
al. (1994) <u>J. Exp. Med.</u> 180:1195-1205. This state of
unresponsiveness may also be induced in vitro, in long-term T
cell clones by stimulation with agents that mimic T cell

5   receptor occupancy, in the absence of CD28 signaling, and/or by
inhibition of IL-2 secretion by T cells. De Silva, et al.
(1991) <u>J. Immunol.</u> 147:3261-3267; Jenkins, et al. (1990) <u>J.
Immunol.</u> 144:16-22; Schwartz (1990) <u>Science</u> 248:1349-1356; and
Sloan-Lancaster, et al. (1994) <u>J. Exp. Med.</u> 180:1195-1205. The

10   observations that high levels of IL-10 are associated with
transplantation tolerance, and that this cytokine has been
shown to inhibit both the antigen presenting and accessory
function of monocytes and the IL-2 production by T cells,
suggest that IL-10 may be involved in the induction of anergy

15   in $CD4^+$ T cells.

In the present study, it is demonstrated that IL-10 is
able to induce a long-lasting antigen-specific unresponsiveness
against allogeneic antigens that cannot be reversed by IL-2 or
CD28 stimulation.

20   <u>Cells</u>

Peripheral blood mononuclear cells were prepared by
centrifugation over Ficoll-hypaque. $CD4^+$ T cells were purified
by negative selection. Negative purification was performed
using a cocktail of antibodies directed against non-$CD4^+$ T

25   cells: CD8, CD14, CD16, CD19, CD20, CD56, HLA-DR. Cells were
incubated with saturating amounts of antibodies for 20 min at
4° C. After washing, Dynabeads (Dynal, Oslo, Norway) were
added at a ratio of 10 beads/target cell and incubated for 1 hr
at 4° C. Beads and contaminating cells were removed by

30   magnetic field. The remaining cells were resuspended with the
same number of beads and a second incubation period for 1 hr at
4° C was performed. After removing contaminating cells, $CD4^+$ T
cells were analyzed by FACScan (Becton Dickinson, Mountain
View, CA) and revealed to be more than 90-95% positive.

35   Monocytes were purified using the same procedure with a
cocktail containing antibodies for: CD2, CD3, CD8, CD16, CD19,
CD20, CD56. These monocytes were >95% $CD14^+$ by FACScan
analysis. In some experiments, $CD4^+$ T were purified by
positive selection using magnetic beads directly coated with

CD4 mAbs, according to the manufacturer instructions (Dynal, Oslo, Norway). With this procedure cells were more than 95% pure.

Reagents

5   Purified recombinant IL-10 was provided by Schering-Plough Research Institute (Kenilworth, NJ). The anti-IL-2 receptor monoclonal antibody B-B10 (Herve, et al. (1990) Blood 75:1017-1023), anti-CD3 mAb SPV-T3 (Spits, et al. (1983) Hybridoma 4:423-437), and anti-CD28 (Becton Dickinson, Mountain View,

10   USA) were previously described. Non-conjugated, PE- or FITC-conjugated antibodies for CD2, CD3, CD4, CD8, CD14, CD16, CD19, CD20, CD56, HLA-DR, and controls mAbs of the appropriate isotypes were purchased from Becton Dickinson (Mountain View, CA).

15

Proliferation assays

       In these proliferation assays, cells were cultured in Yssel's medium (Yssel, et al. (1984) J. Immunol. Methods 72:219-227) supplemented with 10% FCS and 1% human serum. For

20   MLR, purified $CD4^+$ T cells (5 x 10^4 cells/well) were stimulated with purified allogeneic monocytes (5 x 10^4 cells/well) or with irradiated PBMC (10^5 cells/well) in 200 µl flat bottomed 96 well plates (Falcon, Becton Dickinson, Lincoln Park, NJ). PBMC used as stimulators were irradiated at 4,000 rad.

25   For crosslinked anti-CD3 mAb activation, 500 ng/ml of anti-CD3 mAbs diluted in 0.1 M Tris buffer pH 9.5 was incubated for a week at 4° C in flat bottom 96 or 24 well plates. These experimental conditions were found to be optimal in experiments in which different concentrations of antibodies and different

30   incubation times were tested. After washing the plates three times, $CD4^+$ T cells were added at 5 x 10^4 cells/well.

       For activation with phorbol ester (PMA) + Ca^{2+} ionophore (A23187 Sigma, USA), cells were cultured at 5 x 10^4 cells/well and activated for 3 days with PMA (1 ng/ml) and A23187 (500

35   ng/ml).

       To measure the proliferation of T cells, cells were cultured for 72 h or 5 days for MLR experiments at 37° C in 5% CO_2, and subsequently pulsed with $[^3H]$-TdR for 12 h, and

harvested as described in Bacchetta, et al. (1994) _J. Exp. Med._
179:493-502.

Induction of anergy

 To induce anergy, CD4^{+} T cells were cultured at 2.5×10^5
5   cells/ml in 24 well plates (Linbro, ICN Biomedical, Ohio) and
activated either with purified allogeneic monocytes or with
crosslinked anti-CD3 mAbs in the presence of IL-10 (100 U/ml).
After different incubation periods (ranging from 3 to 10 days,
see below) cells were collected, layered on a Ficoll-hypaque
10   gradient to remove dead cells, washed twice, and restimulated
with irradiated allogeneic PBMC or cross-linked anti-CD3 mAbs.

Immunofluorescence analysis

 For detection of cell surface antigens, 10^5 cells were
labeled with PE- or FITC-conjugated mAbs. Cells were incubated
15   for 30 min with the appropriate antibody at 4° C in PBS with
0.1% BSA and 0.02 mM NaN_3. After 3 washes, the labeled cell
samples were analyzed on a FACScan (Becton Dickinson).

Determination of lymphokine production

 Cells were stimulated by cross-linked anti-CD3 mAbs, by
20   PMA and Ca^{2+} ionophore (A23187), or by allogeneic monocytes for
24 hr. The secretion of IL-2, IL-4, IL-5, IL-10, TNF-α, IFN-γ,
and GM-CSF was measured by immunoenzymetric assays as described
ion Bacchetta, et al. (1994) _J. Exp. Med._ 179:493-502 and
Bacchetta, et al. (1995) _Blood_ 85:1944-953. The sensitivity of
25   the various ELISAs was: 20 pg/ml for IL-2; 40 pg/ml for IL-4
and IL-5; 50 pg/ml for GM-CSF and IL-10; and 100 pg/ml for IFN-
γ and TNF-α.

Calcium mobilization studies

 Mobilization of intracellular calcium in anergic cells
30   loaded with indo-1/AM was assessed by using standard
fluorometry. Cells were loaded with 2 μM indo-1/AM in complete
growth medium at 20° C for 45 min. Cells were then washed,
resuspended in Na-HBSS (in mM: 2 $CaCl_2$, 145 NaCl, 5 KCl, 1
$MgCl_2$, 5 d-glucose, 20 HEPES; pH 7.3) containing 1% BSA, and
35   maintained at 20° C for up to two hours. Approximately 5×10^5
cells were then suspended in 2 ml Na-HBSS and maintained at 37°
C in a constantly stirred acrylic cuvette. Anti-CD3 mAbs (1
μg/ml) were added, followed by goat anti-mouse immunoglobulin
to crosslink the anti-CD3 mAbs on the cell surface. No Ca^{2+}

fluxes were detected in the absence of crosslinking, neither in
anergic nor in control cells. Fluorescence measurements to
determine $[Ca^{2+}]_i$ were made using a Photon Technologies Inc.
spectrofluorimeter.

5   IL-10 induces alloantigen-specific T-cell anergy

        As shown previously (Bejarano, et al. (1992) Int. Immunol.
4:1389-1397; and above), IL-10 partially inhibits the
proliferation of peripheral blood CD4$^+$ T cells in response to
allogeneic monocytes in a primary MLR (Fig. 8A). This
10   inhibition was comparable to that induced by anti-IL-2 α chain
receptor mAbs (anti-CD25 mAbs). To determine whether prolonged
incubation of the alloantigen-stimulated CD4$^+$ T cells in IL-10
might also downregulate their proliferative responses following
restimulation, the cells were kept in culture in the presence
15   or absence of IL-10 for 10 days (Fig. 8B). No significant cell
death was observed in either culture condition and comparable
cell numbers were harvested at termination of the assays.
After this culture period, CD4$^+$ control T cells stimulated with
alloantigens in the absence of IL-10 reached a resting state,
20   and could be fully reactivated by the same allogeneic
irradiated PBMC in a secondary MLR, or by third party
allogeneic monocytes in a primary MLR (Fig. 8B). In contrast,
CD4$^+$ T cells incubated in the presence of IL-10 failed to
proliferate in a secondary MLR in response to the same
25   allogeneic PBMC, but they retained their capacity to
proliferate normally to third party alloantigens in a primary
MLR. This unresponsive state could not be reversed by
saturating concentrations of exogenous IL-2 (20 U/ml), or by
anti-CD28 mAbs (10 µg/ml). Furthermore, this state of T-cell
30   unresponsiveness is specific for IL-10 and is not due to
inhibition of the primary MLR, since CD4$^+$ T cells incubated in
the presence of anti-CD25 mAbs (10 µg/ml) for 10 days exhibited
proliferative responses that were comparable to those of their
untreated counterparts.

35   IL-10 induces an anergic state in CD4$^+$ T cells activated by
anti-CD3 mAbs

        Next was analyzed whether the IL-10-induced anergy
reflected a direct effect of IL-10 on CD4$^+$ T cells. For this
purpose, highly purified peripheral blood CD4$^+$ T cells were

activated by crosslinked anti-CD3 mAbs, in the presence or
absence of exogenous IL-10 for 10 days. A direct effect of IL-
10 on CD4$^+$ T cells proliferation was observed (de Waal Malefyt,
et al. (1993) J. Immunol. 150:4754-4765; and Taga, et al.
5 (1993) Blood 81:2964-2971) and this inhibitory effect was
comparable to that induced by anti-CD25 mAbs (Fig. 9A).
Activation of the CD4$^+$ T cells in the presence of IL-10
resulted in a profound state of T-cell unresponsiveness, which
could not be reversed by exogenous IL-2, or anti-CD28 mAbs
10 (Fig. 9B). However, anergic cells proliferated normally in
response to stimulation by Ca^{2+} ionophore and PMA. These
results indicate that IL-10 induces an anergic state in CD4$^+$ T
cells, but that these cells respond normally to signals
circumventing TCR activation. This induction of anergy is IL-
15 10-specific, similarly to IL-10 induced T-cell unresponsiveness
to alloantigens, and not related to inhibition of cell
proliferation, since CD4$^+$ T cells incubated in the presence of
anti-CD25 mAbs for 10 days proliferated normally in response to
restimulation with crosslinked anti-CD3 mAbs. The induction of
20 anergy by IL-10 was dose-dependent with maximal effects
observed at 100 U/ml (Fig. 10). Cells that had been activated
by anti-CD3 mAbs and cultured in the presence or absence of IL-
10 for 10 days were in a resting state and no apoptoic cell
death was observed in the control or IL-10 treated cell
25 cultures. Thus, IL-10 induces a comparable state of T-cell
anergy in alloantigen-specific and anti-CD3 stimulated CD4$^+$ T
cells.

Kinetics of anergy induction by IL-10
        To determine the kinetics of induction of T-cell anergy,
30 CD4$^+$ T cells were activated with crosslinked anti-CD3 mAbs in
the presence or absence of IL-10 and restimulated at different
time points after initiation of the cultures (Fig. 11). CD4$^+$ T
cells cultured in the presence of IL-10 for only 3 days already
failed to proliferate in response to reactivation by
35 crosslinked anti-CD3 mAbs. Interestingly, the anergic state of
these cells could still be reversed by exogenous IL-2 (20 U/ml)
or anti-CD28 (10 μg/ml). In contrast, incubation of the T
cells with IL-10 for 9-10 days resulted in a complete state of
anergy, which could not be reversed, either by the addition of

IL-2 or anti-CD28 mAbs. These results indicate that T cells activated through their TCR/CD3 complexes in the presence of IL-10 acquire different degrees of unresponsiveness, depending on the time periods they had been exposed to IL-10. This

5   indicates that the period of IL-10 exposure will be very important in the effectiveness of suppression.

IL-10 induced T-cell anergy is long-lasting

    To determine the duration of the IL-10 induced anergic state in CD4^{+} T cells after removal of IL-10, the cells were

10 activated with crosslinked anti-CD3 mAbs in the presence or absence of IL-10 for 10 days. After this incubation period, the cells were washed and recultured for 24 days in the presence of low concentrations (2 U/ml) of exogenous IL-2, for optimal maintenance of the T cells over prolonged culture

15 periods, but in the absence of IL-10. These T cells were collected every other day during the culture period, washed, and restimulated with anti-CD3 mAbs. T cells that previously had been incubated with IL-10 failed to proliferate even 24 days after removal of IL-10. In contrast, control CD4^{+} T cells

20 that had been activated by anti-CD3 mAbs in the absence of IL-10, and maintained in the absence of IL-10 for 24 days, proliferated normally in response to stimulation with anti-CD3 mAbs. In addition, the unresponsiveness observed after culturing the IL-10 treated cells for 24 days could not be

25 reversed by IL-2 (20 U/ml) or anti-CD28 (10 μg/ml) mAbs. These results indicate that IL-10 induced T-cell anergy is profound and long lasting.

Anergized T cells fail to secrete cytokines

    T-cell anergy is generally defined as a failure of T cells

30 to proliferate and to produce IL-2 in response to triggering of the TCR (see Schwartz (1990) Science 248:1349-1356). To determine whether T cells rendered unresponsive following activation with either allogeneic monocytes, or anti-CD3 mAbs, in the presence of IL-10 retained their ability to secrete

35 cytokines, their cytokine production after restimulation with the same allogeneic monocytes or anti-CD3 mAbs, respectively, were analyzed.

    CD4^{+} T cells were activated with either crosslinked anti-CD3 mAbs (10 μg/ml), allogeneic monocytes in the absence

(control cells) or presence (anergic cells) of IL-10 (100
U/ml). Cells were kept in culture for 10 days, washed, and
restimulated with crosslinked anti-CD3 mAbs; or TPA (1 pg/ml)
and Ca^{2+} ionophore (A23187: 500 ng/ml); or allogeneic PBMC,
respectively. Supernatants were harvested after 24 hr and the
levels of cytokines were analyzed by ELISA. To measure cell
proliferation, cells were pulsed with [^{3}H] TdR for 12 h at the
end of day 3 for cells activated with crosslinked anti-CD3
mAbs, or at the end of day 5 for cells activated with
allogeneic monocytes.

Table 5 shows the cytokine profile, after restimulation,
of IL-10 induced anergic T cells. T cells which were rendered
unresponsive following activation by either alloantigens plus
IL-10, or anti-CD3 mAbs plus IL-10, failed to produce
detectable amounts of IL-2, IL-4, IL-5, IL-10, TNF-α, IFN-γ,
and GM-CSF at 24 hours or 48 hours after restimulation with the
relevant allogeneic monocytes or anti-CD3 mAbs. In contrast,
the untreated control cells produced levels of IL-2, IL-5, IL-
10, IFN-γ, TNF-α, and GM-CSF that were comparable to those of a
panel of CD4$^+$ T cells derived from different donors and
stimulated in similar fashion. However, these cells did not
secrete detectable levels of IL-4.

Table 5

		cpm	IL-2 pg/ml	IL-4 pg/ml	IL-5 pg/ml	IL-10 pg/ml	TNF-α ng/ml	IFN-γ ng/ml	GM-CSF pg/ml
CD3	control cells	42,369	875 ±59	<40	80 ±23	1,067 ±226	10.5 ±0.9	5.5 ±0.6	1,245 ± 687
	anergic cells	2,635	<40	<40	<20	<50	<0.1	<0.1	<50
TPA + Ca^{2+} ionophore	control cells	147,217	2,121 ±317	<40	841 ±127	51 ±27	21.6 ±5.1	7.6 ±3.1	2,721 ±635
	anergic cells	169,321	1,921 ±427	<40	1,517 ±415	117 ±4.3	18.4 ±3.6	8.4 ±2.6	1,615 ±317
Monocytes	control cells	32,236	617 ±42	<40	64 ±15	897 ±125	9.2 ±0.5	2.3 ±0.1	987 ±98
	anergic cells	869	<40	<40	<20	<50	<0.1	<0.1	<50

Fig. 9 shows that activation with PMA and Ca^{2+} ionophore
completely reversed IL-10 induced-unresponsiveness of anti-CD3
activated T cells.   Table 5 shows that activation of anergic T
cells by PMA and Ca^{2+} ionophore for 24 hrs also resulted in
5   levels of IL-2, IL-5, IL-10, IFN-γ, and TNF-α production that
were comparable to those of their non-anergized counterpart.
Anergic T cells fail to express CD25 after activation

Phenotypic analysis of T cells rendered anergic following
activation by anti-CD3 mAbs in the presence of IL-10 revealed
10   no major differences as compared to their untreated
counterparts, with the exception of a decrease in the IL-2R α
chain (CD25) expression on the anergic T cells (see Figs. 13
and 14A and 14B).   No modulation of CD3 or CD28 expression was
observed on anergic cells, and activation by crosslinked anti-
15   CD3 mAbs enhanced CD3, CD28, and class II MHC expression to the
same extent as on control cells (Fig. 13).   These data exclude
the possibility that the T-cell unresponsiveness was related to
defective TCR/CD3 or CD28 expression and show that signaling
through the TCR/CD3 complex, which is insufficient for T-cell
20   proliferation, still occurs in these anergized T cells.

However, in contrast to the control cells, the anergic T
cells failed to upregulate IL-2R α chain expression, indicating
that inhibition of IL-2R α chain expression is a specific
property of anergized T cells (Fig. 14A and 14B).   The defect
25   in upregulation of CD25 expression was observed also in the
presence of exogenous IL-2, and correlated with the failure of
IL-2 to reverse T-cell anergy induced by 8-10 days of
incubation with IL-10 (Fig. 11).   In contrast, T cells rendered
unresponsive following incubation with IL-10 for 3 days still
30   expressed considerable levels of CD25 (Fig. 14A).   Slight
upregulation of CD25 was also observed following restimulation
by anti-CD3 mAbs, but the levels of expression were still
reduced as compared to untreated cells.   However, addition of
IL-2, which was able to reverse the anergic state, resulted in
35   comparable levels of CD25 expression on control T cells and T
cells incubated in the presence of IL-10 for 3 days (Fig. 14A).
Anti-CD3 mAbs induce normal Ca^{2+} fluxes in anergic T cells

The notion that signaling through the TCR/CD3 complex
occurs in anergic T cells was confirmed by measuring the

induction of Ca^{2+} fluxes in these cells. After loading with Indo-1, cells were activated with anti-CD3 mAbs crosslinked by goat anti-mouse immunoglobulin, as indicated in Fig. 15. Ca^{2+} fluxes induced in anergized T cells following activation with

5   crosslinked anti-CD3 mAbs were comparable to those in untreated control T cells (Fig. 15). The loading of the anergic and control cells with Indo-1 was equivalent, as shown by the comparable rise of Ca^{2+} fluxes induced in the cells by addition of Ca^{2+} ionophore (Fig. 15). These results indicate that IL-10

10 induced anergy affects downstream events involved in T-cell activation.

The present study shows that human peripheral blood CD4$^+$ T cells activated by allogeneic monocytes in the presence of IL-10 for 10 days were rendered unresponsive in an antigen-

15 specific fashion. These unresponsive CD4$^+$ T cells failed to proliferate and to produce cytokines when restimulated by the same allogeneic monocytes. However, these T-cell populations proliferated normally in response to third party alloantigens. A comparable state of T-cell anergy was induced following

20 activation of CD4$^+$ T cells by anti-CD3 mAbs in the presence of IL-10. The IL-10 induced anergy was dose-dependent and lasted for more than 24 days after withdrawal of IL-10, which was the maximal time period analyzed. Furthermore the anergic state could not be reversed by exogenous IL-2, or the addition of

25 anti-CD28 mAbs. In addition, T-cell anergy could not be induced by stimulation with allogeneic monocytes, or anti-CD3 mAbs in the presence of anti-IL-2 receptor mAbs. These results indicate that T-cell anergy is specifically induced by IL-10 and is not simply related to down regulation of IL-2 production

30 by these cells.

IL-10 induced T-cell anergy differs clearly from T-cell unresponsiveness induced by incubation of mouse or human T cells with antigenic peptides in the absence of professional APC. See, e.g., Sloan-Lancaster, et al. (1994) _J. Exp. Med._

35 180:1195-1205; Lamb, et al. (1983) _J. Exp. Med._ 157:1434-1447; and Fasler, et al. (1995) _J. Immunol._ 155:4199-4206. In this latter case, the expression of the TCR/CD3 complex was down-regulated and no mobilization of $[Ca^{2+}]_i$ was observed (Lamb, et al. (1983) _J. Exp. Med._ 157:1434-1447; and Fasler, et al.

(1995) J. Immunol. 155:4199-4206), unlike the findings with IL-10 induced anergy.

On the other hand, CD4+ T cells anergized by IL-10 share many characteristics with anergic T cells described in murine
5   models of anergy induced by lack of costimulatory signals. See De Silva, et al. (1991) J. Immunol. 147:3261-3267; Schwartz (1990) Science 248:1349-1356; Suzuki, et al. (1995) Int. Immunol. 71:37-43; and Ramsdell and Fowlkes (1992) Science 257:1130-1134. Both these forms of anergic cells failed to
10   proliferate and to produce IL-2, but they had a normal expression of CD3 or CD28 surface molecules, and normal calcium fluxes after mobilization of the TCR/CD3 complex. See Schwartz (1990) Science 248:1349-1356. However, T-cell anergy induced by IL-10 is much more profound than that described in these
15   murine models, since the proliferative response of anergic T cells cannot be restored by addition of IL-2 or anti-CD28 mAbs. Furthermore, not only IL-2 production, but also the production, upon subsequent stimulation, of IFN-γ, IL-5, IL-10, TNF-α, and GM-CSF by these anergic cells was impaired. Therefore,
20   induction of T-cell anergy by IL-10 is not simply due to inhibition of IL-2 production and prevention of productive CD28-CD80/CD86 interactions. IL-10 anergized T cells are also different from T cells that undergo apoptosis or anergized T cells able to secrete cytokines in the absence of
25   proliferation. See Jenkins (1992) Immunol. Today 132:69-73; and Groux, et al. (1993) Eur. J. Immunol. 23:1623-1629. In fact, no significant cell loss by apoptosis was observed in the anergic T cells cultured in IL-10 and the anergic T cells were viable as proven by the normal proliferation after stimulation
30   with Ca^{2+} ionophore and PMA.

Overall, these data indicate that signaling through the TCR/CD3 complex is selectively impaired in IL-10 anergized T cells. This is not due to a down regulation of the TCR or CD28 molecules, since the anergic T cells expressed levels of
35   TCR/CD3 and CD28 that were comparable to those of untreated T cells. Furthermore, although stimulation of the anergic T cells through the TCR/CD3 complex did not result in cell proliferation and cytokine production, a clear increase in CD3, CD28, and class II MHC expression was observed following

restimulation of the cells with anti-CD3 mAbs, indicating that some degree of TCR activation occurred in these anergic cells.

This notion was further supported by the observation that Ca^{2+} fluxes in anergized T cells were normal following CD3 activation, demonstrating that calcineurin mediated signaling is not affected. Importantly, a complete reversal of anergy was observed after activation with PMA and Ca^{2+} ionophore. These stimuli, which circumvent TCR activation, completely restored proliferation and cytokine secretion by anergic cells. These findings suggest that IL-10 interferes with proximal events in the TCR signaling pathway, probably at the level of Ras-MAP kinase activation, as is suggested by mouse models of anergy induction. See, e.g., Kang, et al. (1992) Science 257:1134-1138; Fields, et al. (1996) Science 271:1276-1278; and Li, et al. (1996) Science 271:1272-1275. Alternatively, although the signaling cascade downstream of p21ras seems to be intact, it cannot be excluded that IL-10 induces a negative regulator of this signaling pathway.

T cells rendered anergic by activation and incubation with IL-10 for 9-10 days failed, in contrast to their untreated counterparts, to upregulate the IL-2R α chain expression when restimulated with anti-CD3 mAbs. It is therefore tempting to speculate that this defect in upregulation of IL-2R α chain expression accounts for the failure of exogenous IL-2 to reverse anergy. This hypothesis is supported by the observation that T cells rendered anergic by activation and incubation with IL-10 for 3 days, and which still expressed CD25, could be rescued by exogenous IL-2. A comparable, but less profound, defect in IL-2 utilization has been reported for superantigen induced T-cell unresponsiveness in TCR-transgenic mice. See Bhandoola, et al. (1993) J. Immunol. 151:2355-2367. Taken together, these data suggest that anergy induction may be permanent if induced properly.

Among the mechanisms responsible for induction of tolerance to alloantigens a nondeletional mechanism that results in functional inactivation of the appropriate alloreactive T cells has been proposed. This functional inactivation can be achieved by blocking costimulatory signals provided by accessory molecules expressed on APC and T cells.

See Lenschow and Bluestone (1993) Curr. Opin. Immunol. 5:747-
752; Linsley, et al. (1992) Science 257:792-795; Charlton, et
al. (1991) Immunol. Cell. Biol. 692:89-93; and Nakao, et al.
(1994) J. Immunol. 153:5819-5825. Prevention of the

5   interaction between CD28 and either CD80 or CD86 appears to be
critical for this induction of anergy, but blockade of other
costimulatory molecules such as CD2, CD4, or CTLA-4 may also be
involved. See Boussiotis, et al. (1994) J. Exp. Med. 180:1665-
1673. However, the fact that the tolerance induced in these

10 experimental models is long-lasting and potentially
irreversible suggests underlying mechanisms which may be more
complex than the mere lack of a second signal to the T helper
cell upon recognition of alloantigens. It is possible that
some form of active suppression is mediated by cytokines.

15 These cytokines may contribute to the induction or maintenance
of T-cell anergy not only by down-regulating costimulatory
molecules, but also by inducing the expression of negative
regulators, such as CTLA-4. See Tivol, et al. (1995) Immunity
3:541-547. In humans, SCID patients are one of the few

20 examples in which in vivo tolerance is obtained after HLA-
mismatched transplantation. This tolerance is due to a
nondeletional mechanism that is responsible for the functional
inactivation of T cells specifically recognizing the host
alloantigens. See above, and Roncarolo, et al. (1988) J. Exp.

25 Med. 167:1523-1534; and Bacchetta, et al. (1993) J. Clin.
Invest. 91:1067-1078. These host-reactive T cells secrete high
levels of IL-10 in vitro, and high IL-10 levels have been
observed in vivo, suggesting that IL-10 may play a role in the
induction and maintenance of tolerance. See Bacchetta, et al.

30 (1994) J. Exp. Med. 179:493-502. Based on the present results
it is tempting to conclude that the high levels of IL-10
observed in these patients render the host-reactive T cells
anergic in vivo. High levels of IL-10 secretion prior to
transplantation have been also shown to correlate with a

35 successful outcome of the transplant (Holler, et al. (1995)
Eur. J. Cancer 31:39-45), further supporting the hypothesis
that IL-10 might play a role in the induction of tolerance.

Collectively, these data suggest that IL-10 has an
important role in transplantation tolerance by inducing anergy

to donor-specific and/or host-specific alloantigens. In addition, they suggest that IL-10 will have a potential clinical utility in preventing or reducing GVHD and allograft rejection.

5   Fig. 16 shows that the proliferative responses of purified CD4$^+$ T cells that had been rendered anergic following stimulation with crosslinked anti-CD3 mAbs (100 ng/ml) in the presence of IL-10 (100 U/ml) for 10 days, can be partially restored following restimulation with very high concentrations
10   of crosslinked anti-CD3 mAbs.

        Table 6 shows the cytokine profile of anergic cells upon restimulation with anti-CD3 mAbs. Cells (2.5 x 10^6 cells/ml) were stimulated with crosslinked anti-CD3 mAb (100 ng/ml) in the presence (anergic cells) or absence (control cells) of IL-
15   10 (100 U/ml). After 10 days, cells were collected, layered over a Ficoll-Hypaque gradient, washed 3 times, and restimulated at 2.5 x 10^5 cells/ml with crosslinked CD3 mAb (either 10 mg/ml or 700 mg/ml). Twenty-four hours later, supernatants were collected and cytokine amounts were measured
20   by ELISA. Results are mean ± SD of multiple measurements of one representative experiment. From the data set forth in Table 6, it is seen that restimulation of CD4$^+$ T cells that have been anergized following stimulation by crosslinked anti-CD3 mAbs in the presence of IL-10 for 10 days with extremely
25   high levels of anti-CD3 mAbs (700 mg/ml), results in low levels of proliferation and in a Tr1 cytokine production profile, which is characterized by the production of high levels of IL-10, very low levels of IL-2 and no detectable levels of IL-4. In addition, IL-5, IFN-γ, GM-CSF, and TNF-α production can be
30   detected. In contrast, the anergized CD4$^+$ T cells failed to proliferate and to produce cytokines when stimulated with crosslinked anti-CD3 mAbs at concentrations of 10 μg/ml.

Table 6

		cpm	IL-2 pg/ml	IL-4 pg/ml	IL-5 pg/ml	IL-10 pg/ml	TNF-α ng/ml	IFN-γ ng/ml	GM-CSF pg/ml
CD3	control cells	42,369	875 ± 59	<40	80 ± 23	1067 ± 226	10.5 ± 0.9	5.5 ± 0.6	1245 ± 687
10 µg/ml	anergic cells	2,635	<40	<40	<20	<40	<20	<40	<40
CD3	control cells	75,236	1045 ± 105	<40	107 ± 39	1145 ± 321	11.2± 1.3	4.3 ± 1.2	1569 ± 537
700 µg/ml	anergic cells	24,569	321 ± 65	<40	445 ± 51	6421 ± 547	9.5 ± 0.8	1.6 ± 0.4	1125 ± 327

5

Fig. 17 shows the proliferative responses of Tr1 clones as compared to those of Th1 and Th2 clones.

Table 7 shows the cytokine production profile of Tr1, Th0, Th1, and Th2 clones obtained from anergic cell populations of 2

10   different donors. The various CD4^{+} T-cell clones were collected 12 days after stimulation with an irradiated feeder cell mixture + PMA and restimulated by a mixture of anti-CD3 and anti-CD28 mAbs for 24 hrs. Cytokine production (mean ± SD) is expressed as pg/10^6 cells/ml. The characteristic cytokine

15   production profile of Tr1 clones is shown: no significant production of IL-2 and IL-4, but very high levels of IL-10 production. In addition, these cells produce IL-5 and IFN-γ and TNF-α. This cytokine production profile is clearly different from that of the Th0, Th1, and Th2 clones obtained

20   from anergized or non-anergized control T-cell populations.

Table 7

	no. clones	IL-2	IL-4	IL-5	IL-10	IFN-γ	TNF-α
Anergic							
Tr1	15	10	10	1070	12290	3870	5950
Th0	5	530	2270	6120	660	1990	2340
Th2	5	10	3250	13690	1340	350	1140
Th1	5	520	10	80	790	6330	6310
Control							
Th0	5	490	2900	11240	850	2656	4210
Th2	5	10	4890	22210	1690	440	520
Th1	5	470	10	10	470	3040	2120

25

Figs. 18A and 18B show that IL-10 production by Tr1 clones occurs rapidly after activation of the cells.

Example 9:   Human Tr1 cell clones
Isolation of purified CD4+ T cells

        Peripheral blood mononuclear cells were prepared by
centrifugation over Ficoll-hypaque. CD4$^+$ T cells were purified
5   by negative selection. Negative purification was performed
using a cocktail of antibodies directed against non-CD4$^+$ T
cells: CD8, CD14, CD16, CD19, CD20, CD56, HLA-DR. Cells were
incubated with saturating amounts of antibodies for 20 min at
4° C. After washing, Dynabeads (Dynal, Norway) were added at a
10   ratio of 10 beads/target cell and incubated for 1 hr at 4° C.
Beads and contaminating cells were removed by magnetic field.
The remaining cells were resuspended with the same amount of
beads and a second incubation period for 1 hr at 4° C was
performed. After removing contaminating cells, CD4$^+$ T cells
15   were analyzed by FACScan (Becton Dickinson, Mountain View, CA)
and revealed to be more than 90-95% positive. In some
experiments, CD4$^+$ T were purified by positive selection using
magnetic beads directly coated with CD4 mAbs, according to the
manufacturer's instructions (Dynal, Norway). With this
20   procedure cells were more than 95% pure.
Culture conditions

        In these assays, cells were cultured in Yssel's medium
supplemented with 10% FCS and 1% human serum.
        For crosslinked anti-CD3 mAbs activation, 100 µg/ml (for
25   cloning anergic cells) or 500 ng/ml (for induction of anergy)
of anti-CD3 mAbs (SPV-T3) diluted in 0.1 M Tris buffer, pH 9.5,
was incubated for a week at 4° C in flat bottom 96 wells
plates. Then, the plates are washed three times with RPMI.
Induction of anergy and cloning
30   To induce anergy, CD4$^+$ T cells were cultured at 2.5×10^5
cells/ml in 24 well plates (Linbro, ICN Biomedical, Ohio) and
activated with crosslinked anti-CD3 mAbs in the presence of IL-
10 (100 U/ml). After 10 days, cells were collected, layered
on a Ficoll-hypaque gradient to remove dead cells, washed
35   twice, and cloned at 0.3 cells/well. End point dilution
cloning was performed by diluting anergic cells in feeder (5 x
10^5 PBMC + 5×10^4 EBV transformed B cells (JY) plus 2 U/ml of
IL-2 on plates previously coated with 100 µg/ml of anti-CD3
mAbs. After 10 days, fresh medium containing 2 U/ml of IL-2

was added in each well. Every 3 weeks, all wells were
reactivated with feeders (10^6 PBMC + 10^5 EBV-LCL) + PHA (10
µg/ml) + IL-2 (2U/ml). After 5-8 weeks, cells that grow out
were transferred in 48 well plates and then to 24 well plates.

5   Maintenance of Tr1 cells

T cell clones were stimulated every 3 weeks with feeders
(10^6 PBMC + 10^5 EBV-LCL) + PHA (10 µg/ml) + IL-2 (2 U/ml).
Every 3 days cells were checked, split if necessary, and fresh
medium containing 2 U/ml of IL-2 was added.

10   Thus, the present invention provides Tr1 cells, purified
to homogeneity, e.g., in a clonal form, or populations
comprising Tr1 cells at high levels, including at least about
40%, generally at least about 50%, typically at least about
60%, more typically at least about 70%, preferably at least
15   about 80%, more preferably at least about 90%, and in
especially preferred embodiments, at least about 95% or higher
purity of Tr1 type cells.

Example 10.   In vitro differentiation of novel regulatory CD4+
20   T cells in both human and mouse

Induction and maintenance of peripheral tolerance are
important mechanisms to maintain the balance of the immune
system. In addition to T-cell tolerance and T-cell anergy,
active suppression mediated by T-cell or T-cell factors has
25   been proposed as a mechanism for peripheral tolerance. Germain
and Benacerraf (1981) Scand. J. Immunol. 13:1. However, thus
far, the inability to isolate and clone regulatory T cells
involved in antigen-specific inhibition of immune responses has
hampered the elucidation of the mechanisms underlying active
30   suppression. IL-10 has been associated with the induction and
maintenance of tolerance in SCID patients following allogeneic
stem cell transplantation. Bacchetta, et al. (1994) J. Exp.
Med. 179:493-502. In addition, stimulation of human CD4+ T
cells in the presence of IL-10 results in antigen-specific T-
35   cell unresponsiveness. Groux, et al. (1996) J. Exp. Med.
184:1-11. Here it is shown that activation of both human and
murine CD4+ T-cells in the presence of IL-10 gives rise to CD4+
T cell clones with low proliferative capacity, producing high
levels of IL-10, low IL-2 and no IL-4. These antigen-specific

T cell clones suppressed the proliferation of naive CD4$^+$ T
cells in response to the same antigen. This inhibitory effect
was mediated by both IL-10 and TGF-β. Thus, IL-10 drives the
generation of a novel CD4$^+$ T-cell subset designated T

5   regulatory cells (Tr1) which suppress immune responses and are
probably recruited to induce and maintain tolerance.

One of the primary features of the immune system is the
discrimination of self from non-self. Rocha and von Boehmer
(1991) Science 251:1225-8; Nossal (1987) Int. Rev. Immunol.

10   2:321. Immunologic tolerance provides the immune system with
self/non-self discrimination and is critical to prevent
pathogenic reactivities against self antigens. The immune
system is confronted with repetitive stimulations by non
pathogenic antigens through inhalation and ingestion of foreign

15   substances. To avoid chronic cell activation and inflammation,
the immune system must develop unresponsiveness to such
stimuli. Therefore, mechanisms must exist that regulate
inappropriately reactive T cells in the peripheral compartment.
Several mechanisms of peripheral immune tolerance have been

20   described, including T-cell anergy (Jenkins, et al. (1990) J.
Immunol. 144:16-22; and Schwartz (1990) Science 248:1349-56),
T-cell deletion (Rocha and von Boehmer (1991) Science 251:1225-
8), and active immune suppression (Germain and Benacerraf
(1981) Scand. J. Immunol. 13:1).

25   Understanding these mechanisms of tolerance induction is
clinically relevant both for the treatment of autoimmune
diseases and in transplantation, where the graft must be
ultimately recognized as self, and be tolerated. In humans,
SCID patients are one of the few examples in which in vivo

30   tolerance is established after HLA mismatched stem cell
transplantation. Bacchetta, et al. (1993) J. Clin. Invest.
91:1067-78. It was previously shown that this tolerance is due
to a non deletional mechanism that is responsible for the
functional inactivation of T cells specifically recognizing the

35   host alloantigens. Roncarolo, et al. (1988) J. Exp. Med.
167:1523-34. These host reactive CD4$^+$ T cells generally have a
low proliferative capacity and produce very low levels of IL-2
after antigenic stimulation. In contrast, they secreted high
levels of IL-10, and high levels of IL-10 mRNA were also

observed in the PBMC of these patients as compared to normal
controls. Moreover, high levels of IL-10 secretion before
transplantation have been shown to correlate with successful
and uneventful clinical course after the transplant. Holler,
5   et al. (1994) Annual meeting of the EBMT, Davos, Switzerland.
In addition, it was shown that IL-10 induces a long lasting
antigen-specific anergic state in human CD4$^+$ T cells. These
observations support the hypothesis that IL-10 might play a
role in the induction of tolerance.

10   IL-10 induced anergic T cells fail to proliferate and to
produce cytokines in response to antigenic stimulation, or to
crosslinked anti-CD3 mAb. Groux, et al. (1996) J. Exp. Med.
184:1-11.

       For example, IL-10 induced T-cell anergy was obtained in
15   human CD4$^+$ T cells. In brief, CD4$^+$ T cells were cultured at
10^6 cells/ml in 24 wells plates and activated with either
crosslinked anti-CD3 mAb (SPV-T3; 100 ng/ml) or purified
irradiated allogeneic monocytes (10^6 cells/ml), in the presence
of IL-10 (100 U/ml). After 10 days, cells were collected,
20   layered on a ficoll gradient, and centrifuged for 30 min at
1200 rpm, to remove dead cells. The viable cells were
collected from the interface, washed twice, and cloned. The
cloning was performed by FACS sorting. Cells were labeled with
an anti-CD4-FITC mAb (Becton, Dickinson) and the viable cells
25   were sorted at one cell/well in wells coated with anti-CD3
(SPV-T3-100 µg/ml) in 100 µl. After sorting, a mixture of
irradiated feeders cells (EBV-LCL 10^5 cell/ml and PBMC 10^6/ml)
and 10 U/ml of recombinant IL-2 in 100 µl were added. Clones
were expanded with IL-2 (10 U/ml) and by repetitive
30   restimulation with high doses of crosslinked anti-CD3 mAb (100
µg/ml).

       Here it is shown that very high doses of crosslinked anti-
CD3 mAb (700 µg/ml) resulted in a partial restoration of the
proliferative response of CD4$^+$ T cells rendered anergic by IL-
35   10, however, these responses were still 3-5 fold lower than
those obtained with their non-anergized counterparts (Table 9).
Reversion of anergy with high doses of antigen has also been
described for anergized murine CD4$^+$ Th1 cells. Schwartz (1990)
Science 248:1349-56. Interestingly, the cytokine production

profile of the anergic human T cells following stimulation with
high doses of crosslinked anti-CD3 mAb did not fit the Th0,
Th1, or Th2 phenotype (Table 9). Mosmann and Coffman (1989)
Ann. Rev. Immunol. 7:145-73. The most striking characteristic

5   of these cells was their unusually high level of IL-10
production. In addition, they secreted essentially no IL-4 and
very low levels of IL-2, whereas their levels of IL-5, IFN-γ,
TGF-β, and TNF-α production were comparable to those of human
Th0 clones (Table 9).

10   Table 9 shows cytokine production by human Tr1 cells. CD4^{+} T
cells were stimulated with crosslinked anti-CD3 mAb (100 ng/ml) in the
presence (anergic cells) or absence (control cells) of IL-10 (100
U/ml). After 10 days of culture, cells cultured with IL-10 were
anergic when restimulated with anti-CD3 mAbs (100 ng/ml). However,

15   activation of these IL-10 anergized CD4^{+} T cells with high
concentration of crosslinked anti-CD3 mAb (700 μg/ml) partially
restored cell proliferation (24,569 cpm). However, the proliferative
response of the control cells cultured for 10 days in the absence of
IL-10 was always higher (75,236 cpm). Levels of cytokine production

20   were measured by ELISA in the culture supernatants of these two
populations of cells activated with crosslinked anti-CD3 mAbs (700
μg/ml) for 48 hrs. Results represent data from one representative
experiment and are expressed in pg/ml.

       The cells from these 2 populations were cloned and after

25   expansion, the different clones were stimulated by crosslinked anti-
CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) mAbs. The cytokine production
was analyzed by ELISA in culture supernatants collected after 48 hrs
of culture. Results represent mean values of data obtained from
individual T-cell clones.

30   To generate antigen-specific T-cell clones, CD4^{+} T cells from
donor JDV were stimulated with irradiated purified allogeneic
monocytes in the presence or absence of IL-10 (100 U/ml). After 10
days CD4^{+} T cells were cloned. The different T-cell clones were
expanded and selected for their specificity against the same

35   allogeneic monocytes. To measure cytokine production, T cells were
activated with crosslinked anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml)
mAbs, and supernatants were collected after 48 hrs of culture or 72
hrs for TGF-β. The results set forth in Table 9 represent pooled data
from various independent experiments.

Table 9

T-cell lines or clones	number of clones	IL-2	IL-4	IL-5	IL-10	TNF-α	IFN-γ	TGF-β
cells	preinc	ubated	with	IL-10				
anergic cell line	1	320	<40	440	6,410	9,520	1,690	
Tr1	15	<20	<40	1,060	12,230	5,980	3,830	
Th0	5	530	2,268	6,120	630	2,370	1,920	
Th2	5	<20	3,245	13,680	1,320	1,110	320	
Th1	5	520	<40	80	850	6,360	6,380	
JDV15	1	<20	<40		11,620		1,370	220
JDV24	1	<20	<40		14,370		420	230
JDV308	1	70	<40		12,630		720	510
cells	preinc	ubated	with	IL-10				
control cell line	1	1,040	<40	110	1,120	11,210	4,320	
Th0	5	480	2,903	11,240	820	4,200	2,630	
Th2	5	<20	4,894	22,210	1,780	510	410	
Th1	5	470	<40	110	430	2,120	3,020	
JDV305	1	140	<40		<40		2,520	220

5   Analysis of T-cell clones isolated from a human CD4^{+} T
cell population that was initially stimulated by crosslinked
anti-CD3 mAb (10 μg/ml) in the presence of IL-10 revealed that
half of the CD4^{+} T cell clones analyzed also displayed a
similar cytokine profile characterized by high levels of IL-10,
10   very low levels of IL-2, and no IL-4 secretion (Table 9).
       Human CD4^{+} T cell clones or populations at 10[6] cell/ml
were stimulated by cross-linked anti-CD3 mAbs (10 μg/ml) and
anti-CD28 (1 μg/ml) for 48 hr. The production of IL-2, IL-4,
IL-5, IL-10, TNF-α, and IFN-γ was measured by immunoenzymatic
15   assays. See Murphy, et al. (1996) J. Exp. Med. 183:901-13.
The cytokine production of IL-2, IL-5, IL-10, and IFN-γ by
murine CD4^{+} T cell clones was measured in supernatants
collected after 48 hrs of culture of cells (10[6] cells/well)
stimulated with OVA-peptide (1 μM) and irradiated total splenic
20   APCs by ELISA. For TGF-β measurements cells were cultured in
Yssel's medium without serum and the amount of TGF-β was

measured using a commercial ELISA kit (R&D System) after an
acid activation, according to the manufacturer's instruction.

5
In contrast, only Th1, Th2, and Th0 clones were isolated
from control T-cell populations that were cultured in the
absence of IL-10 (Table 9). Thus activation of human CD4+ T
cells in the presence of IL-10 results in the generation of a
new T-cell subset with a unique cytokine profile and low
proliferative capacity. Based on their function these cells
are designated in the earlier filing Th3 cells, and

10
redesignated here Tr1 cells.

Both alloantigen-specific Tr1 CD4+ clones (JDV 24) and non
alloantigen-specific Tr1 clones (JDV 15 and JDV 308) were
isolated from a CD4+ T-cell population that was initially
stimulated with allogeneic monocytes in the presence of IL-10

15
(Table 9). In contrast, no Tr1 clones, but only Th0 (JDV 305)
and Th1 clones were isolated from a control population of CD4+
T cells initially stimulated by allogeneic monocytes in the
absence of IL-10, further demonstrating that IL-10 drives the
differentiation of CD4+ T cells towards a Tr1 phenotype.

20
Tr1 cells can also be isolated from mice. OVA-specific
CD4+ T cells obtained from OVA-specific TCR transgenic mice
(Murphy, et al. (1990) Science 250:1920-1722) that had been
repeatedly stimulated in the presence of IL-10, or a
combination of IL-10 and IL-4 displayed the cytokine profile of

25
Tr1 cells. See Murphy, et al. (1996) J. Exp. Med. 183:901-13.

Flow cytometry analysis of intracellular cytokines
synthesis. Analysis of intracellular cytokines by flow
cytometry was performed. Kuhn, et al. (1993) Cell 75:263-274.
Cells (10^6 cell/ml) were activated with crosslinked anti-CD3

30
and anti-CD28 mAbs (Pharmingen) for 4 hrs. Two hours before
harvesting, Brefeldin A was added at 10 µg/ml. Cells were
harvested, washed, and fixed with formaldehyde (2%). For
intracellular staining cells were incubated with the following
mAbs: anti-IL-4-FITC or PE (11B11; 5 µg/ml), anti-IFN-γ-PE

35
(XMG1.2-PE; 5 µg/ml), anti-IL-5-PE (TRFK5; 2.5 µg/ml), anti-IL-
10-FITC (JES-16E3; 5 µg/ml), anti-IL-2-PE (JES6-5H4; 2.5 µg/ml)
or the anticlonotype KJ1-26-FITC (5 µg/ml). Samples were
analyzed on a FACScan™ (Becton Dickinson).

These cells were characterized by high levels of IL-10 and
IL-5 secretion and low levels of IL-2 and IL-4 secretion (Fig
19). In contrast, control CD4^{+} T cells isolated from OVA-
specific TCR transgenic mice stimulated in the presence of IL-4
5   alone displayed the typical Th2 type profile, secreting IL-4,
IL-5, and IL-10 (Fig 19). In addition, several OVA-specific
CD4^{+} T clones with the Tr1 cytokine profile were isolated from
a population of T cells that had been repeatedly stimulated in
the presence of IL-10.

10   Table 10 shows cytokine production by murine OVA-specific
CD4^{+} T cell clones derived from IL-10 anergized T cells of
D011-10 TCR-transgenic mice. Naive (MEL14 bright) CD4^{+} T cells
from D011-10 transgenic mice were stimulated with OVA peptide
(0.6 µM) in the presence or absence of IL-10 (100 U/ml). After
15   repetitive stimulations under the same conditions, the CD4^{+} T
cells from these 2 populations were cloned by limiting dilution
at 0.3 cells/well in wells coated with 50 µg/ml of anti-CD3 mAb
in PBS and containing irradiated total splenocytes (10^7
cells/ml) and 20 U/ml IL-2· Clones were expanded with IL-2 (20
20   U/ml) and IL-4 (20 U/ml) and repetitive stimulation with OVA
peptide (2 µM) and irradiated splenic APCs (10^7 cells/ml), in
the presence of IL-2 and IL-4. However, no Tr1 clones were
obtained from T-cell cultures that had been stimulated in the
presence of IL-4. After expansion, the T-cell clones were
25   stimulated by OVA peptide (1 µM) and irradiated total splenic
APCs. Cytokines were analyzed by ELISA in culture supernatants
collected after 48 hrs of culture. For TGF-β measurement cells
were cultured in Yssel's medium without serum for 72 hrs and
the amount of inactive TGF-β was measured by ELISA (R&D
30   Systems) after an acid activation, according to the
manufacturer's instruction. Results represent pooled data from
representative experiments.

Table 10

T-cell clones	initial incubation with IL-10	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)	IFN-γ (ng/ml)	TGF-β (pg/ml)	clone type
A-10-1	yes	<40	<50	1230±310	105±27		Tr1
A-10-9	yes	<40	<50	1020±120	87±17	435±9	Tr1
A-10-11	yes	<40	<50	1850±410	217±68	747±9	Tr1
A-10-14	yes	<40	<50	1820±120	198±67		Tr1
A-10-2	yes	102±26	250±45	850±130	789±124		Th0
A-10-7	yes	97±27	897±102	410±120	267±247		Th0
A-10-15	yes	52±19	89±17	205±80	420±41		Th0
A-10-19	yes	69±27	890±48	310±120	107±57		Th0
A-3	no	<40	1247±157	510±110	<75		Th2
A-5	no	<40	879±87	310±60	<75		Th2
A-7	no	129±48	<50	<75	975±97	715±2	Th1
A-13	no	89±28	598±59	215±30	143±13		Th0
A-14	no	203±49	<50	<75	1249±49		Th1

5　　　　　Human Tr1 cells stimulated with either crosslinked anti-
CD3 and anti-CD28 mAbs or allogeneic monocytes (for JDV 24)
showed reduced proliferative responses as compared to a
representative Th0 clone (JDV 305) used as a control (Figs 20A
and 20B). Proliferation assays were carried out in Yssel's
10　medium (Yssel, et al. (1984) J. Immunol. Meth. 72:219-27)
supplemented with 10% FCS and 1% human serum for human cells or
without human serum for murine cells. Alloantigen-specific
stimulation were performed by stimulating human CD4+ T-cell
clones (10^5 cells/well) with purified irradiated (4,000 rad)
15　monocytes (10^5 cells/well) in 200 μl round bottomed 96 well
plates (Linbro). CD4+ T cell proliferation was measured after
5 days incubation at 37° C and 5% CO_2 and a subsequent 12 h
pulse with ^{3}[H]-TdR after which the cells were harvested as
described in . Activation of human CD4+ T cells with
20　crosslinked anti-CD3 and anti-CD28 was performed as previously
described by Groux, et al. (1996) J. Exp. Med. 184:1-11. In
brief, antibodies were diluted at the indicated concentration
in 0.1 M Tris buffer pH 9.5, and plates were incubated for a
week at 4° C in flat bottom 96 well plates. After washing the
25　plates, CD4+ T cells were added at a concentration of 5 x 10^4

72

cells/well, and proliferation was measured after 3 days
incubation followed by a 12 h pulse with ^{3}[H]-TdR. Murine CD4$^+$
T-cell populations of T-cell clones were stimulated at a
concentration of 5 x 10^4 cells/well with OVA peptide (0.6 μM)
5   and irradiated (4000 rad) total splenocytes (5 x 10^5
cells/well) for 3 days followed by a 12 h pulse with ^{3}[H]-TdR.

A similar impairment in the proliferative responses,
following similar stimulation with OVA peptide and splenic
APCs, was observed with the OVA-specific murine Tr1 clones and
10   the OVA-specific T cell population which had been repeatedly
stimulated in the presence of IL-10 alone, or IL-10 in
combination with IL-4. In contrast, proliferation of a Th1
clone (A-7) or of the OVA-specific Th2 cell population that had
been generated in the presence of IL-4 was in the normal range
15   (Figs 20A and 20B).

Kinetic studies indicated that IL-10 is produced rapidly
after activation of the human Tr1 clones, since the IL-10
protein is already detectable in the supernatant 8 hrs after
activation. In contrast, IL-10 production by Th0, Th1, and Th2
20   clones obtained from the same cloning experiment occurred much
later, and was first detectable 20 hrs after activation. The
rapid IL-10 production by Tr1 clones generally occurs
simultaneously to IL-2 production, or even precedes it. Early
endogenous IL-10 production accounts in part for the low
25   proliferative capacity of the Tr1 clones in response to TCR/CD3
stimulation (Figs 20A and 20B), since proliferation of the Tr1
clones was partially restored by the addition of neutralizing
anti-IL-10 mAbs, which is consistent with observations
indicating that IL-10 prevents or inhibits T cell
30   proliferation. de Waal Malefyt, et al. (1991) J. Exp. Med.
174:915-24. In contrast, anti-IL-10 mAbs had no effect on the
proliferative responses of the Th1, Th2, or Th0 control clones
(Figs 20A and 20B).

Proliferation of Tr1 clones was also partially restored by
35   neutralizing anti-TGF-β mAbs (Figs 20A and 20B), whereas again
the proliferation of Th1 clones remained unaffected (Figs 20A
and 20B). Interestingly, the effect of combination of anti-IL-
10 and anti-TGF-β had additive effects in specifically
restoring the proliferative responses of human of Tr1 clones

(Figs 20A and 20B). Similar data were obtained with mouse Tr1
cells (Figs 20A and 20B), confirming the general principle that
endogenously produced IL-10 and TGF-β play a major role in
controlling the proliferative responses of Tr1 cells.

5   The observation that Tr1 cells secrete high levels of the
immunosuppressive cytokines IL-10 and TGF-β, and low levels of
the T-cell growth promoting cytokines IL-2 and IL-4, suggested
that antigen-specific activation of Tr1 cells may result in
inhibition of antigen-specific proliferation of bystander T
10   cells. To address this question, co-culture experiments using
a transwell system were performed. Resting human CD4+ T cells,
autologous to the different JDV clones, were stimulated with
irradiated allogeneic monocytes of donor N in the bottom
compartment, whereas alloantigen-specific JDV24 and non antigen
15 . specific JDV308 Tr1 clones were stimulated in the transwell
basket with monocytes from donor N. In Figure 21A shows that
alloantigen-specific stimulation of the Tr1 clone JDV24
strongly reduced the proliferative responses of the naive human
CD4+ T cells, whereas the non-antigen specific Tr1 clone JDV
20   308 was ineffective. A slight enhancement of the naive CD4+ T
cell proliferation was noticed following the co-culture with
the antigen-specific Th0 clone (JDV 305) (Fig 21A). Addition
of neutralizing anti-IL-10 and anti-TGF-β partially restored
the proliferative responses of naive CD4+ T cells in the .
25   presence of Tr1 cells, whereas the addition of the two
antibodies had additive effects, and restored the proliferative
responses of the CD4+ T cells by 80%.

        Similar data were obtained with mouse Tr1 clones. In
Figure 21B it is shown that the proliferation of naive CD4+ T
30   cells in response to OVA-peptide and splenic APCs is
dramatically reduced following co-culture with OVA peptide
activated Tr1 clones or with the activated Tr1-cell populations
generated in the presence of IL-10. In contrast, control OVA-
specific Th1 clones and Th2-cell populations generated in the
35   presence of IL-4 had no suppressive effects, but rather .
enhanced the proliferation of CD4+ T cells.

        Again, addition of neutralizing anti-IL-10 or anti-TGF-β
antibodies partially restored the OVA-specific proliferative
response of the mouse T cells, whereas the combination of the

two antibodies had additive effects and restored the
proliferation to 70% of control levels (Fig 21B). These data
demonstrate that the suppressive activities of both human and
mouse Tr1 cells are predominantly mediated by IL-10 and TGF-β.

5   The present findings indicate that stimulation of $CD4^+$ T
cells in the presence of IL-10 generates a novel subset of
$CD4^+$ T cells which can down regulate antigen-specific immune
responses via secretion of IL-10 and TGF-β. The cytokine
profile appears to be stable, since Tr1 clones that had been
10   kept in culture for more than 6 months produced the same
cytokine profile after activation.

        T regulatory cells have been described in several models
of oral tolerance. Chen, et al. (1994) <u>Science</u> 265:1237-1240;
Miller, et al. (1992) <u>Proc. Nat'l Acad. Sci. USA</u> 89:421-5.
15   $CD8^+$ regulatory T cells were isolated in the Lewis rat after
stimulation with myelin basic protein as an oral antigen
(Miller). These regulatory T cells mediated active suppression
by secreting TGF-β. Furthermore, antigen-specific regulatory
$CD4^+$ T-cell clones were isolated from SJL mice following
20   induction of oral tolerance after feeding the animals with
myelin basic protein. Transfer of these $CD4^+$ T-cell clones,
generated after oral tolerance induction, suppressed
experimental allergic encephalomyelitis. This suppression was
relieved by anti-TGF-β, suggesting that also these clones
25   mediated their suppression via TGF-β. Indeed, these T-cell
clones, in contrast to Tr1 cells described here, produced very
high levels of active TGF-β, (up to 1000 pg/ml) whereas low
levels of IL-10 and IL-4 were detected. In addition, T-cell
clones isolated from MBP transgenic mice produced exclusively
30   TGF-β and no IL-10, IL-4, IL-2 and TNF-α. Weiner, et al.
(1996) FASEB Meeting, New Orleans, Louisiana. A 2558. Thus,
these murine T cells, designated Th3 cells, secrete TGF-β as
the primary cytokine which mediates their suppressor activity.
In contrast to Th3 cells, the Tr1 cells described here produce
35   low levels of active TGF-β. Only trace amounts of active TGF-β
(less than 40 pg/ml) were detected in the supernatants of
activated Tr1-cell clones, and the levels of latent TGF-β were
comparable to those of control T-cells (Tables 9 and 10).
Furthermore, these Tr1 cells produce high levels of IL-10 which

contributes to their suppressor activity. Therefore, it seems likely that Tr1 cells may act by both secreting IL-10 and promoting the activation of TGF-β through a still yet to be defined mechanism. All together, these data suggest that Th3

5   and Tr1 cells are two distinct populations of CD4$^+$ regulatory T cells. The former is specifically generated after oral exposure to antigens and acts through TGF-β secretion, the latter may be the result of antigen-specific stimulation in the lymphoid organs and is dependent on IL-10 production. The

10 isolation of similar Tr1 cells from the peripheral blood of SCID patients in which high levels of IL-10 in vivo are associated with tolerance after allogeneic stem cell transplantation, supports this hypothesis and suggests that Tr1 cells may be generated in vivo by exposure of naive T cells to

15 alloantigens in the presence of IL-10. Furthermore, based on the in vitro immunosuppressive activities of Tr1 cells, it is tempting to speculate that these host-reactive Tr1 cells have similar properties in vivo, and therefore are responsible for transplantation tolerance.

20   IL-10 deficient mice develop inflammatory bowel disease, which in young mice is prevented by the administration of IL-10. Kuhn, et al. (1993) Cell 75:263-274. In addition, administration of IL-10 significantly inhibits the development of colitis induced by transferring CD45RB^{hi} CD4$^+$ T cells into

25 SCID mice. Powrie, et al. (1994) Immunity 1:553-562. Colitis induced by CD45RB^{hi} CD4$^+$ T cells in SCID mice could also be prevented by co-transfer of CD45RB^{lo} CD4$^+$ T cells. Powrie, et al. (1993) Int. Immunol. 5:1461-1471; Powrie, et al. (1994) J. Exp. Med. 179:589-600. However, the protective effects was

30 completely ablated by anti-TGF-β mAbs. Powrie, et al. (1996) J. Exp. Med. 183:2669-74. These results demonstrate that there are clear similarities between these regulatory cells preventing colitis and the Tr1 cells described here. It should be pointed out that all the regulatory cells described thus far

35 have been isolated after in vivo priming with antigen. The possibility to generate Tr1 cells after antigen-exposure in vitro may be a major advantage to obtain these cells for further functional analysis and potential clinical applications.

Collectively these data demonstrate that IL-10, both in
mice and human, induces the generation of Tr1 cells which
downregulate antigen-specific T cell responses and therefore
are potentially important for the general induction and
5   maintenance of tolerance.

Applicants have deposited separate cultures of E. coli
MC1061 carrying pH5C, pH15C, and pBCRF1(SRα) with the American
Type Culture Collection, Rockville, MD, USA (ATCC), on December
20, 1989, under accession numbers 68191, 68192, and 68193,
10   respectively. These deposits were made under conditions as
provided under ATCC's agreement for Culture Deposit for Patent
Purposes, which assures that the deposit will be made available
to the U.S. Commissioner of Patents and Trademarks pursuant to
35 U.S.C. §122 and 37 C.F.R. §1.14, and will be made available
15   to the public upon issue of a U.S. patent, which requires that
the deposit be maintained. Availability of the deposited
strain is not to be construed as a license to practice the
invention in contravention of the rights granted under the
authority of any government in accordance with its patent laws.
20   The descriptions of the foregoing embodiments of the
invention have been presented for purpose of illustration and
description. They are not intended to be exhaustive or to
limit the invention to the precise forms disclosed, and
obviously many modifications and variations are possible in
25   light of the above teaching. The embodiments were chosen and
described in order to best explain the principles of the
invention to thereby enable others skilled in the art to
utilize the invention in various embodiments and with various
modifications as are suited to the particular use contemplated.
30   It is intended that the scope of the invention be defined by
the claims appended hereto.

SEQUENCE LISTING

5   (1) GENERAL INFORMATION:

     (i) APPLICANT: Schering Corporation

    (ii) TITLE OF INVENTION: Use of Interleukin-10 to Produce a
10   Population of Suppressor Cells

   (iii) NUMBER OF SEQUENCES: 25

    (iv) CORRESPONDENCE ADDRESS:
15   (A) ADDRESSEE: Schering-Plough Corporation
            (B) STREET: 2000 Galloping Hill Road
            (C) CITY: Kenilworth
            (D) STATE: New Jersey
            (E) COUNTRY: USA
20   (F) ZIP: .07033

    (v) COMPUTER READABLE FORM:
            (A) MEDIUM TYPE: Floppy disk
            (B) COMPUTER: Macintosh
25   (C) OPERATING SYSTEM: 7.5.3

   (vii) CURRENT APPLICATION DATA:
            (A) APPLICATION NUMBER:
            (B) FILING DATE:
30   (C) CLASSIFICATION:

   (vii) PRIOR APPLICATION DATA:
            (A) APPLICAITON NUMBER: US 08/643,810
            (B) FILING DATE: 06-MAY-1996
35   (A) APPLICATION NUMBER: US 07/846,208
            (B) FILING DATE: 04-MAR-1992
            (C) CLASSIFICATION:

  (viii) ATTORNEY/AGENT INFORMATION:
40   (A) NAME: Foulke, Cynthia L.
            (B) REGISTRATION NUMBER: 32,364
            (C) REFERENCE/DOCKET NUMBER: DX0261K1

    (ix) TELECOMMUNICATION INFORMATION:
45   (A) TELEPHONE: 908-298-2987
            (B) TELEFAX: 908-298-5388

    (2) INFORMATION FOR SEQ ID NO:1:
50

    (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 178 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
55   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

60

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

        Met His Ser Ser Ala Leu Leu Cys Cys Leu Val Leu Leu Thr Gly Val
        1   5   10   15

```
            Arg Ala Ser Pro Gly Gln Gly Thr Gln Ser Glu Asn Ser Cys Thr His
                         20                  25                  30
```

5

```
            Phe Pro Gly Asn Leu Pro Asn Met Leu Arg Asp Leu Arg Asp Ala Phe
                         35                  40                  45
```

```
            Ser Arg Val Lys Thr Phe Phe Gln Met Lys Asp Gln Leu Asp Asn Leu
                         50                  55                  60
```

10

```
            Leu Leu Lys Glu Ser Leu Leu Glu Asp Phe Lys Gly Tyr Leu Gly Cys
                         65                  70                  75             80
```

```
            Gln Ala Leu Ser Glu Met Ile Gln Phe Tyr Leu Glu Glu Val Met Pro
                         85                  90                  95
```

15

```
            Gln Ala Glu Asn Gln Asp Pro Asp Ile Lys Ala His Val Asn Ser Leu
                        100                 105                 110
```

20

```
            Gly Glu Asn Leu Lys Thr Leu Arg Leu Arg Leu Arg Arg Cys His Arg
                        115                 120                 125
```

```
            Phe Leu Pro Cys Glu Asn Lys Ser Lys Ala Val Glu Gln Val Lys Asn
                        130                 135                 140
```

25

```
            Ala Phe Asn Lys Leu Gln Glu Lys Gly Ile Tyr Lys Ala Met Ser Glu
                145                 150                 155                 160
```

```
            Phe Asp Ile Phe Ile Asn Tyr Ile Glu Ala Tyr Met Thr Met Lys Ile
                                165                 170                 175
```

30

```
            Arg Asn
```

35   (2) INFORMATION FOR SEQ ID NO:2:

```
               (i) SEQUENCE CHARACTERISTICS:
                   (A) LENGTH: 170 amino acids
                   (B) TYPE: amino acid
40                 (C) STRANDEDNESS: single
                   (D) TOPOLOGY: linear

              (ii) MOLECULE TYPE: peptide
```

45

```
              (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
```

```
            Met Glu Arg Arg Leu Val Val Thr Leu Gln Cys Leu Val Leu Leu Tyr
50          1                5                  10                  15
```

```
            Leu Ala Pro Glu Cys Gly Gly Thr Asp Gln Cys Asp Asn Phe Pro Gln
                         20                  25                  30
```

55

```
            Met Leu Arg Asp Leu Arg Asp Ala Phe Ser Arg Val Lys Thr Phe Phe
                         35                  40                  45
```

```
            Gln Thr Lys Asp Glu Val Asp Asn Leu Leu Leu Lys Glu Ser Leu Leu
                         50                  55                  60
```

60

```
            Glu Asp Phe Lys Gly Tyr Leu Gly Cys Gln Ala Leu Ser Glu Met Ile
                65                  70                  75                  80
```

```
        Gln Phe Tyr Leu Glu Glu Val Met Pro Gln Ala Glu Asn Gln Asp Pro
                         85              90                  95
```

5
```
        Glu Ala Lys Asp His Val Asn Ser Leu Gly Glu Asn Leu Lys Thr Leu
                        100             105                 110
```

```
        Arg Leu Arg Leu Arg Arg Cys His Arg Phe Leu Pro Cys Glu Asn Lys
                    115             120                 125
```
10
```
        Ser Lys Ala Val Glu Gln Ile Lys Asn Ala Phe Asn Lys Leu Gln Glu
            130                 135             140
```

15
```
        Lys Gly Ile Tyr Lys Ala Met Ser Glu Phe Asp Ile Phe Ile Asn Tyr
            145                 150             155                 160
```

```
        Ile Glu Ala Tyr Met Thr Ile Lys Ala Arg
                        165                 170
```

20   (2) INFORMATION FOR SEQ ID NO:3:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 160 amino acids
             (B) TYPE: amino acid
25   (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

30

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
        Ser Pro Gly Gln Gly Thr Gln Ser Glu Asn Ser Cys Thr His Phe Pro
35      1               5               10                  15
```

```
        Gly Asn Leu Pro Asn Met Leu Arg Asp Leu Arg Asp Ala Phe Ser Arg
                    20              25                  30
```
40
```
        Val Lys Thr Phe Phe Gln Met Lys Asp Gln Leu Asp Asn Leu Leu Leu
                35                  40                  45
```

```
        Lys Glu Ser Leu Leu Glu Asp Phe Lys Gly Tyr Leu Gly Cys Gln Ala
            50                  55                  60
```
45
```
        Leu Ser Glu Met Ile Gln Phe Tyr Leu Glu Glu Val Met Pro Gln Ala
        65                  70                  75                  80
```

50
```
        Glu Asn Gln Asp Pro Asp Ile Lys Ala His Val Asn Ser Leu Gly Glu
                        85                  90                  95
```

```
        Asn Leu Lys Thr Leu Arg Leu Arg Leu Arg Arg Cys His Arg Phe Leu
                    100                 105                 110
```
55
```
        Pro Cys Glu Asn Lys Ser Lys Ala Val Glu Gln Val Lys Asn Ala Phe
                    115                 120                 125
```

```
        Asn Lys Leu Gln Glu Lys Gly Ile Tyr Lys Ala Met Ser Glu Phe Asp
            130                 135                 140
```
60
```
        Ile Phe Ile Asn Tyr Ile Glu Ala Tyr Met Thr Met Lys Ile Arg Asn
            145                 150                 155                 160
```

(2) INFORMATION FOR SEQ ID NO:4:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 147 amino acids
    (B) TYPE: amino acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: peptide

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Thr Asp Gln Cys Asp Asn Phe Pro Gln Met Leu Arg Asp Leu Arg Asp
 1               5                  10                  15

Ala Phe Ser Arg Val Lys Thr Phe Phe Gln Thr Lys Asp Glu Val Asp
                20                  25                  30

Asn Leu Leu Leu Lys Glu Ser Leu Leu Glu Asp Phe Lys Gly Tyr Leu
            35                  40                  45

Gly Cys Gln Ala Leu Ser Glu Met Ile Gln Phe Tyr Leu Glu Glu Val
        50                  55                  60

Met Pro Gln Ala Glu Asn Gln Asp Pro Glu Ala Lys Asp His Val Asn
 65                  70                  75                  80

Ser Leu Gly Glu Asn Leu Lys Thr Leu Arg Leu Arg Leu Arg Arg Cys
                85                  90                  95

His Arg Phe Leu Pro Cys Glu Asn Lys Ser Lys Ala Val Glu Gln Ile
            100                 105                 110

Lys Asn Ala Phe Asn Lys Leu Gln Glu Lys Gly Ile Tyr Lys Ala Met
         115                 120                 125

Ser Glu Phe Asp Ile Phe Ile Asn Tyr Ile Glu Ala Tyr Met Thr Ile
        130                 135                 140

Lys Ala Arg
 145
```

(2) INFORMATION FOR SEQ ID NO:5:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 15 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (oligonucleotide)

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTAAGGAGGT TTAAC   15

(2) INFORMATION FOR SEQ ID NO:6:

  (i) SEQUENCE CHARACTERISTICS:

                    (A) LENGTH: 10 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear
5
          (ii) MOLECULE TYPE: DNA (oligonucleotide).

10   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

    ATGAGCTCAT   10

15   (2) INFORMATION FOR SEQ ID NO:7:

          (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 60 base pairs
                    (B) TYPE: nucleic acid
20   (C) STRANDEDNESS: double
                    (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (oligonucleotide)

25

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

30
    AGCCCAGGCC AGGGCACCCA GTCTGAGAAC AGCTGCACCC ACTTCCCAGG TAACCGGTAC   60

     (2) INFORMATION FOR SEQ ID NO:8:

35   (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 56 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: double
                    (D) TOPOLOGY: linear
40
        (ii) MOLECULE TYPE: DNA (oligonucleotide)

45   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

    CGGTTACCTG GGAAGTGGGT GCAGCTGTTC TCAGACTGGG TGCCCTGGCC TGGGCT   56

     (2) INFORMATION FOR SEQ ID NO:9:
50
          (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 62 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: double
55   (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (oligonucleotide)

60
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTAACCTGCC TAACATGCTT CGAGATCTCC GAGATGCCTT CAGCAGAGTG AAGACTTTCT   60

TT   62

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 48 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (oligonucleotide)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTTCACTCTG CTGAAGGCAT CTCGGAGATC TCGAAGCATG TTAGGCAG   48

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (oligonucleotide)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CAAATGAAGG ATCAGCTGGA CAACTTGTTC TTAAG   35

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 44 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (oligonucleotide)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTTAAGAACA AGTTGTCCAG CTGATCCTTC ATTTGAAAGA AAGT   44

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 69 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (oligonucleotide)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAGTCCTTGC TGGAGGACTT TAAGGGTTAC CTGGGTTGCC AAGCCTTGTC TGAGATGATC   60

CAGTTTTAT   69

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 73 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (oligonucleotide)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GATCATCTCA GACAAGGCTT GGCAACCCAG GTAACCCTTA AAGTCCTCCA GCAAGGACTC   60

CTAGATAAAA CTG   73

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 61 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (oligonucleotide)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTAGAGGAGG TGATGCCCCA AGCTGAGAAC CAAGACCCAG ACATCAAGGC GCATGTTAAC   60

G   61

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 65 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (oligonucleotide)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTTAACATGC GCCTTGATGT CTGGGTCTTG GTTCTCAGCT TGGGGCATCA CCTCCTCTAG   60

TCGAC   65

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 63 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (oligonucleotide)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AACTCCCTGG GGGAGAACCT GAAGACCCTC AGGCTGAGGC TACGGCGCTG TCATCGATCT   60

GCA   63

(2) INFORMATION FOR SEQ ID NO:18:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 59 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (oligonucleotide)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GATCGATGAC AGCGCCGTAG CCTCAGCCTG AGGGTCTTCA GGTTCTCCCC CAGGGAGTT   59

(2) INFORMATION FOR SEQ ID NO:19:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 60 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (oligonucleotide)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGATTTCTTC CCTGTCAAAA CAAGAGCAAG GCCGTGGAGC AGGTGAAGAA CGCGTGCATG   60

(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 54 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (oligonucleotide)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CACGCGTTCT TCACCTGCTC CACGGCCTTG CTCTTGTTTT GACAGGGAAG AAAT   54

(2) INFORMATION FOR SEQ ID NO:21:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 58 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (oligonucleotide)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CGCGTTTAAT AATAAGCTCC AAGACAAAGG CATCTACAAA GCCATGAGTG AGTTTGAC   58

(2) INFORMATION FOR SEQ ID NO:22:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 45 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (oligonucleotide)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ACTCATGGCT TTGTAGATGC CTTTGTCTTG GAGCTTATTA TTAAA   45

(2) INFORMATION FOR SEQ ID NO:23:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 51 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (oligonucleotide)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

ATCTTCATCA ACTACATAGA AGCCTACATG ACAATGAAGA TACGAAACTG A   51

(2) INFORMATION FOR SEQ ID NO:24:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 38 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double
            (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (oligonucleotide)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AGCTTCAGTT TCGTATCTTC ATTGTCATGT AGGCTTCTAT GTAGTTGATG AAGATGTCAA   60
ACTC   64
        30

(2) INFORMATION FOR SEQ ID NO:25:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 498 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (oligonucleotide)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AATTCATGGA GCGAAGGTTA GTGGTCACTC TGCAGTGCCT GGTGCTGCTT TACCTGGCAC   64

CTGAGTGTGG AGGTACAGAC CAATGTGACA ATTTTCCCCA GACCTAAGAG ATGCCTTCAG   120

TCGTGTTAAA ACCTTTTTCC AGACAAAGGA CGAGGTAGAT AACCTTTTGC TCAAGGAGTC   180

TCTGCTAGAG GACTTTAAGG ATGCCAGGCC CTGTCAGAAA TGATCCAATT CTACCTGGAG   240

GAAGTCATGC CACAGGCTGA AACCAGGACC CTGAAGCCAA AGACCATGTC AATTCTTTGG   300

GTGAAAATCT AAAGACCCTA CGGCTCCGCC TGCGCAGGTG CCACAGGTTC CTGCCGTGTG   360

AGAACAAGAG TAAAGCTGTG AACAGATAA AAAATGCCTT TAACAAGCTG CAGGAAAAAG   420

GAATTTACAA AGCCATGAGT GAATTTGACA TTTTTATTAA CTACATAGAA GCATACATGA   480

CAATTAAAGC CAGGTGAG   498

WHAT IS CLAIMED IS:

1.   A method of manufacturing a medicament for inhibiting, by an immune system, an antigen-specific response, including antigen-specific responses to subsequent presentation of said antigen, comprising admixing an effective amount of interleukin-10 and either said antigen or an anti-CD3 antibody.

2.   The method of claim 1, wherein said effective amount is sufficient to decrease responder T cell activation.

3.   The method of claim 1, wherein said interleukin-10 is human interleukin-10.

4.   A substantially pure antigen-specific anergic T cell characterized by production upon restimulation of:
    a)   low IL-2;
    b)   low IL-5;
    c)   intermediate IFN-γ;
    d)   low GM-CSF; and
    e)   high IL-10.

5.   The T cell of claim 4, which further produces upon restimulation high TNF-α.

6.   The T cell of claim 4, wherein said production of:
    a)   IL-2 is less than about 500 pg/ml;
    b)   IL-5 is between about 300 and 3000 pg/ml;
    c)   IFN-γ is at least about 1000 pg/ml;
    d)   GM-CSF is about 300-3000 pg/ml; and
    e)   IL-10 is at least about 3000 pg/ml.

7.   The T cell of claim 6, wherein said IL-10 level upon restimulation is at least about 5x that of a Th1 cell.

8.   The T cell of claim 4, which exhibits antigen-specific anergy for at least about 21 days.

9.   A method of preparing the substantially pure antigen-specific anergic T cell of claim 4 comprising administering to a precursor of said T cell a combination of:

   i)   exogenous IL-10; and

   ii) either said antigen or anti-CD3 antibodies.

10.   The method of claim 9, wherein said precursor is a CD4$^+$ T cell.

11.   The method of claim 9, wherein said IL-10 is administered for at least about 7 days.

12.   The method of claim 9, wherein said antigen is a protein antigen, a particulate antigen, an alloantigen, an autoantigen, or MHC-associated antigen.

13.   A pharmaceutical composition comprising IL-10, an antigen or an anti-CD3 antibody, and a pharmaceutically acceptable carrier.

14.   The composition of claim 13, wherein said IL-10 is human IL-10.

15.   The composition of claim 13, wherein said antigen is an alloantigen, a self antigen, a protein antigen; a particulate antigen or a MHC-associated antigen.
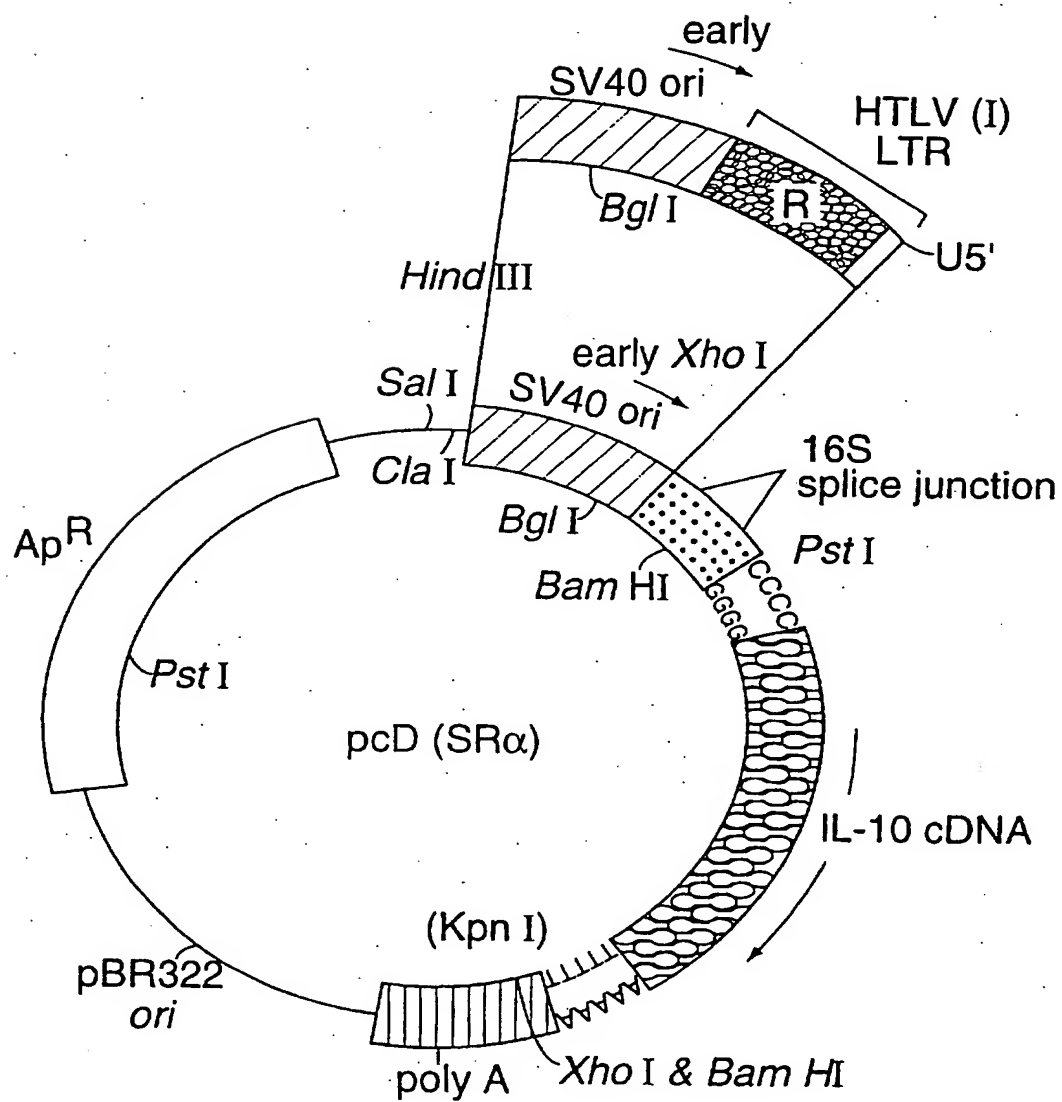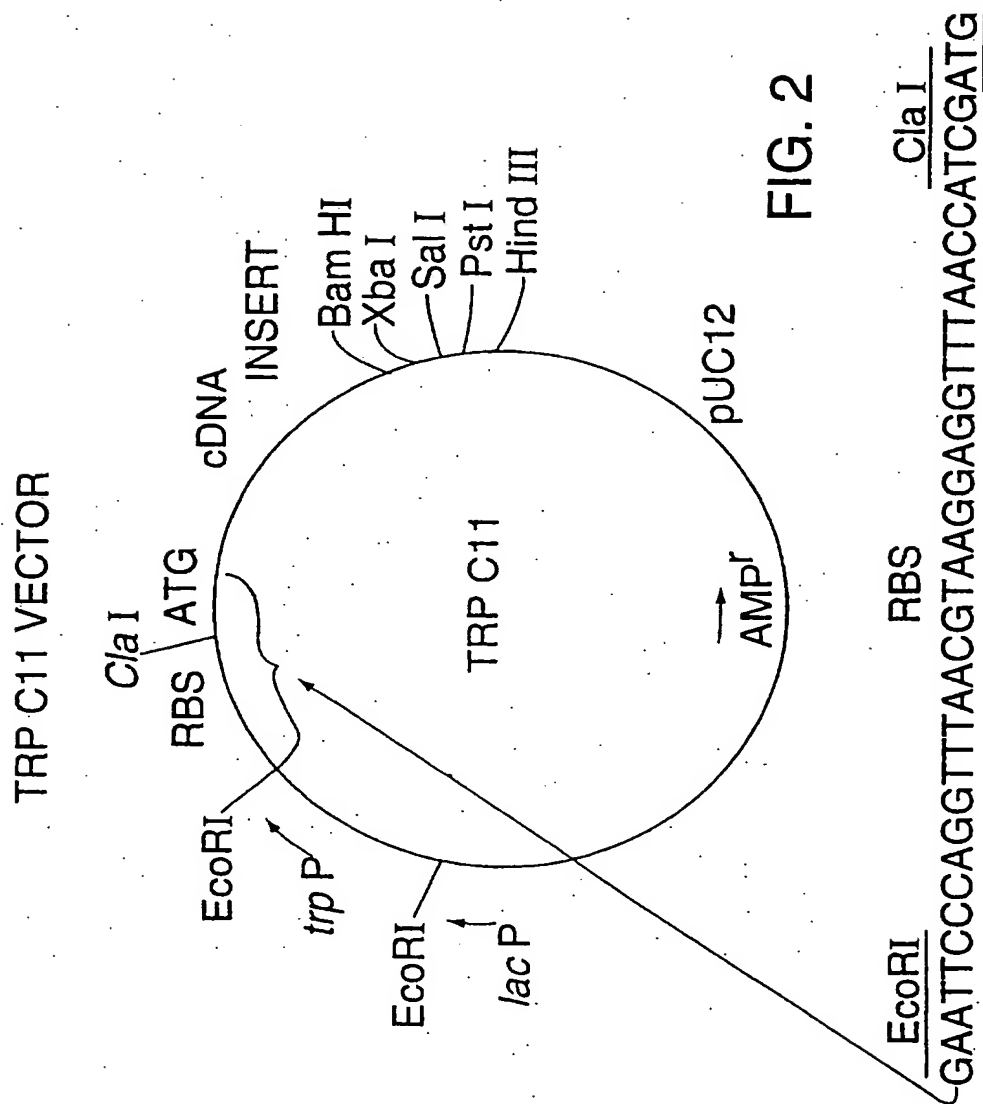
FIG. 1

FIG. 2

c.p.m. x 10^{-3}
^{3}[H]TdR incorporation
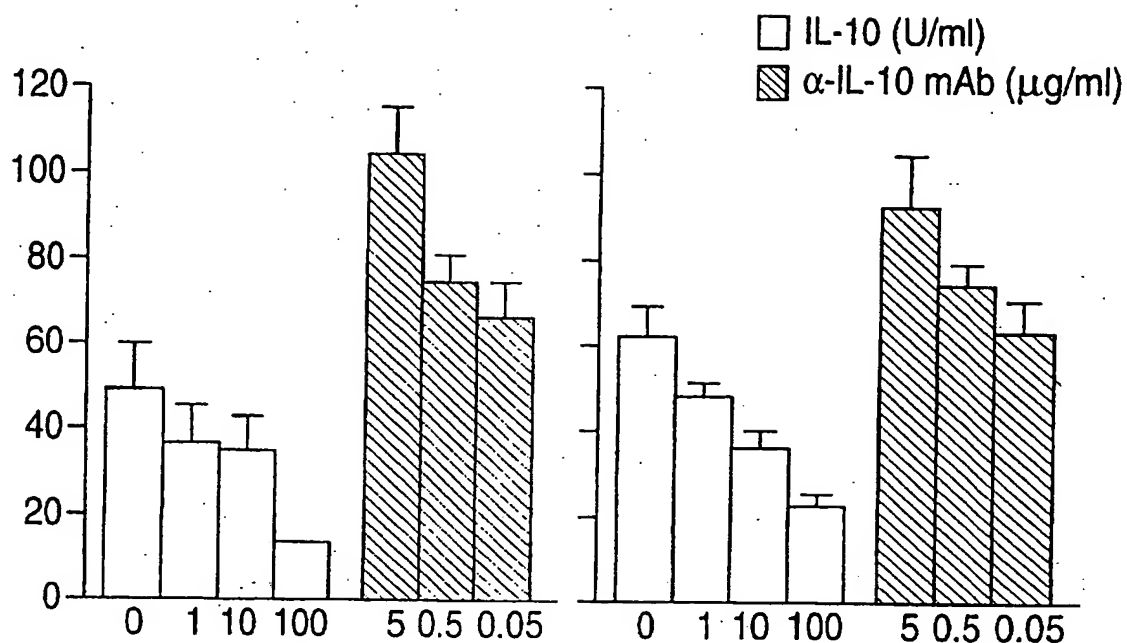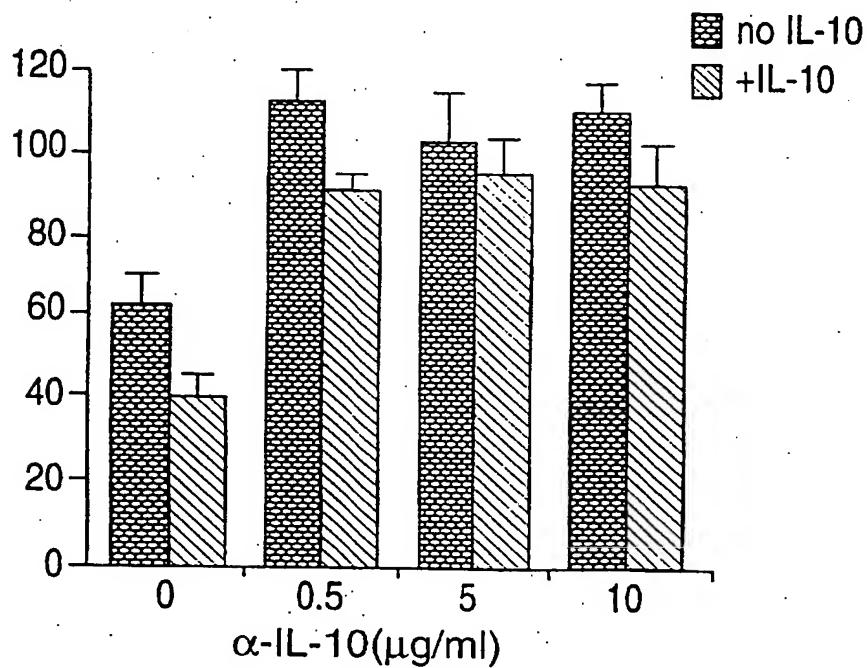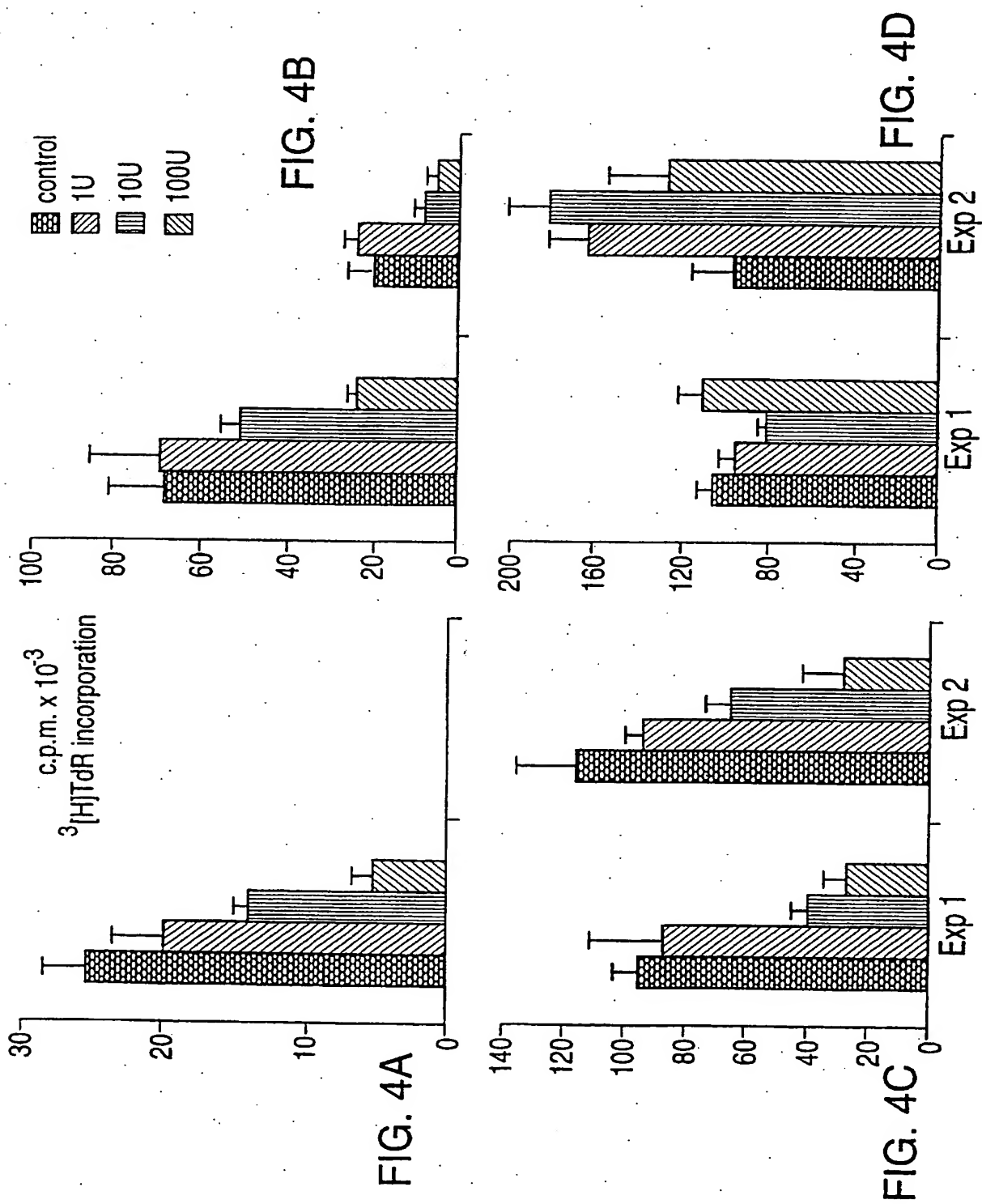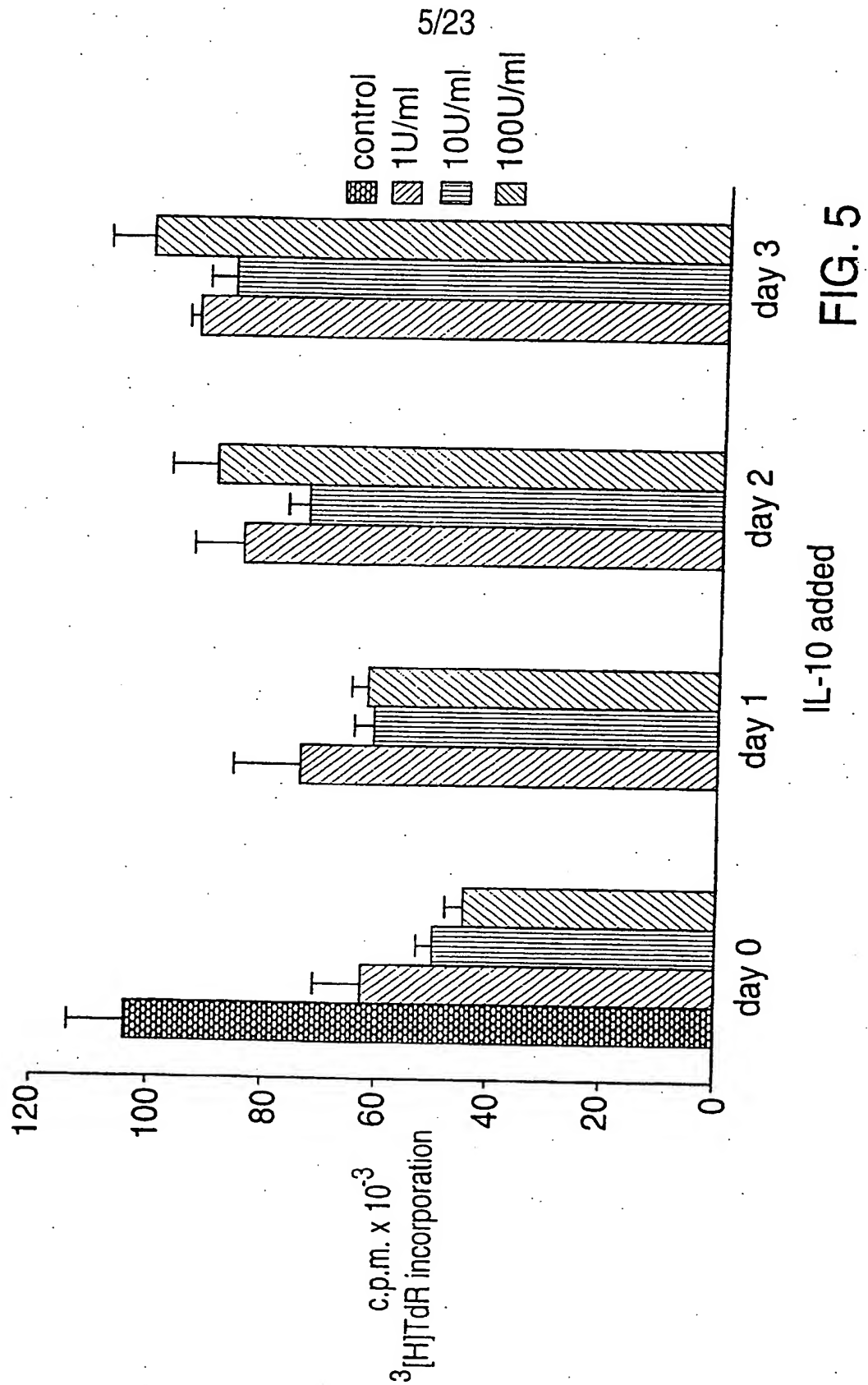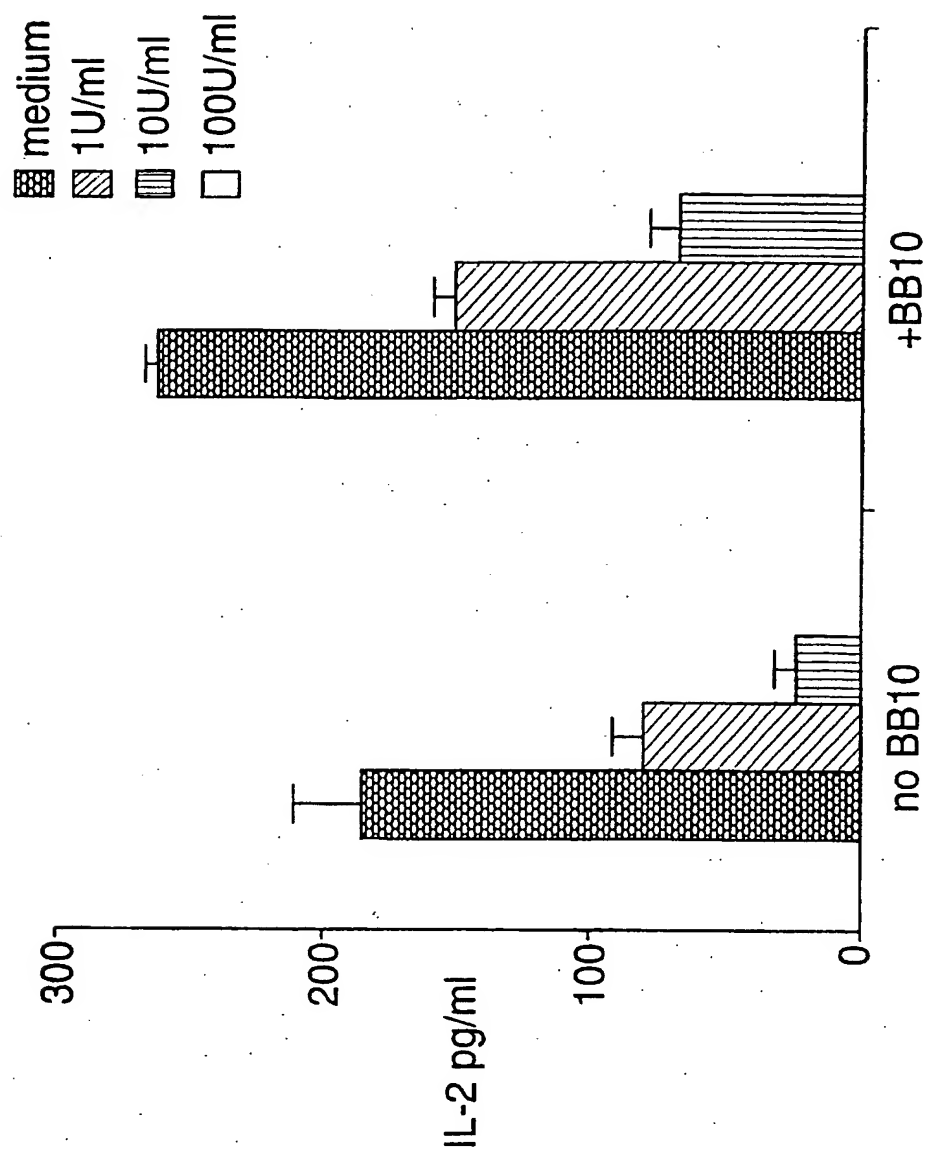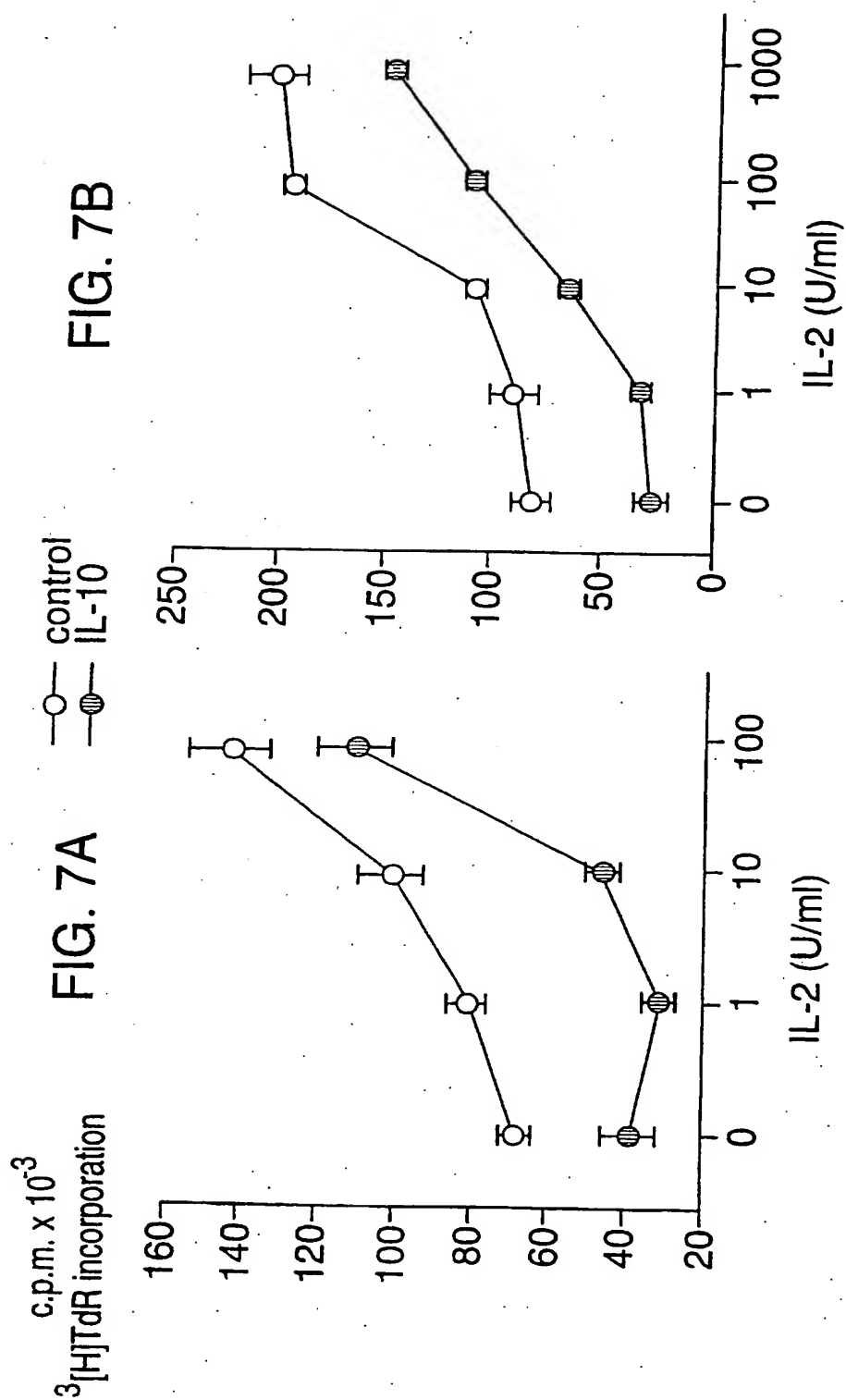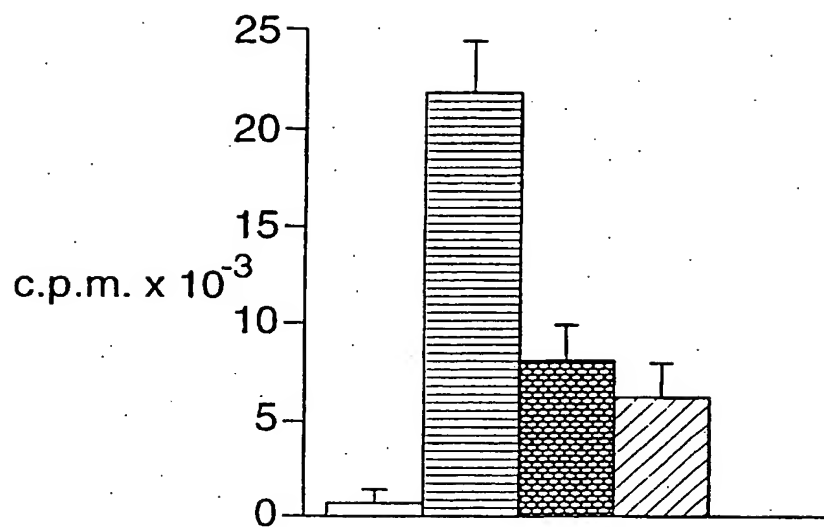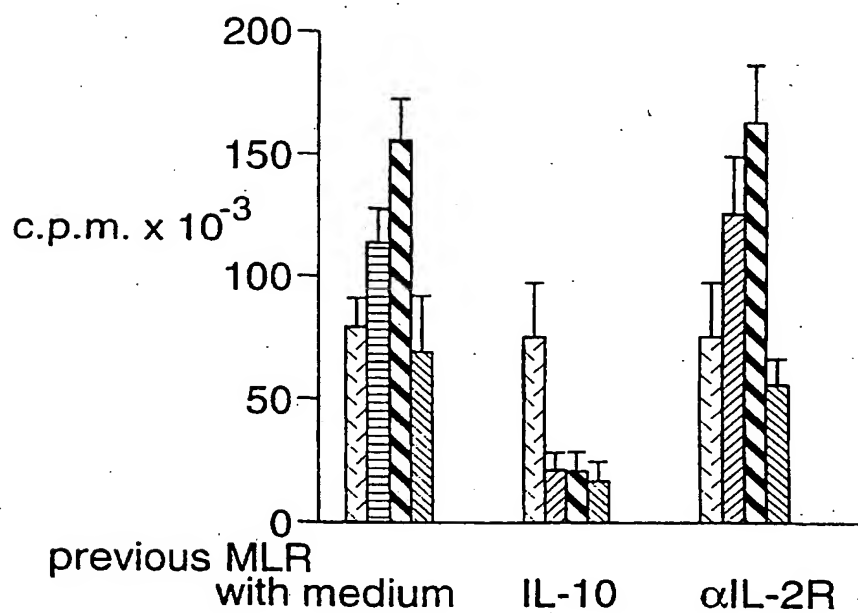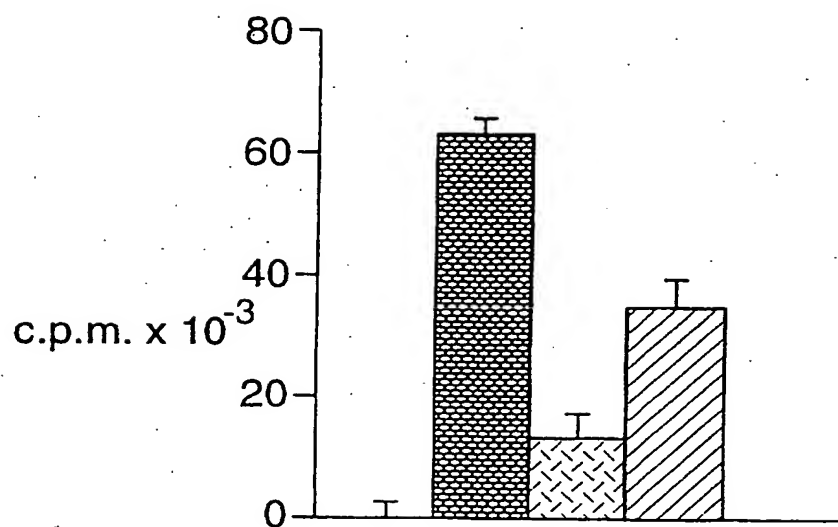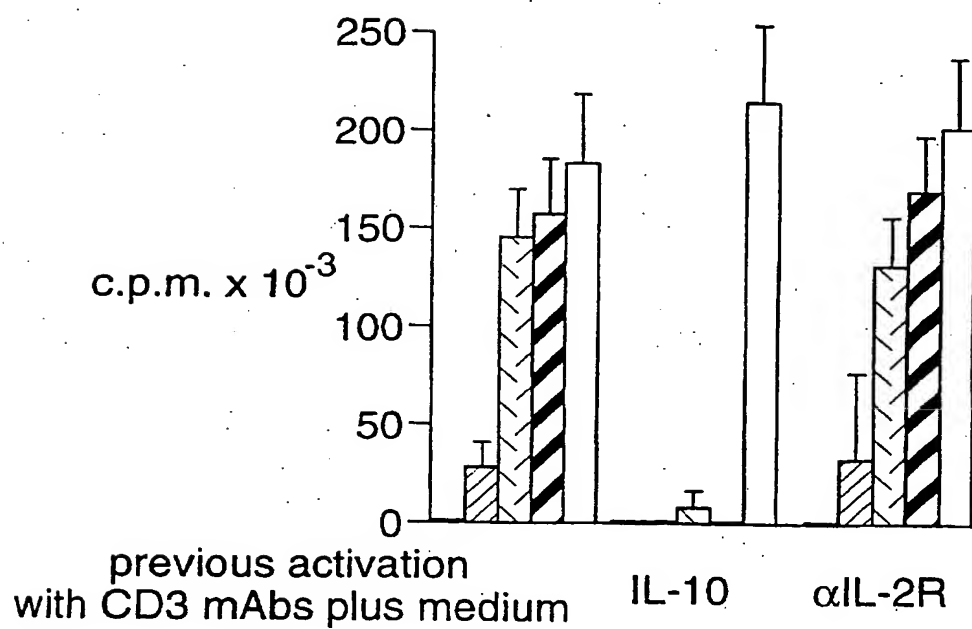
☐ IL-10 (U/ml)
▨ α-IL-10 mAb (μg/ml)

FIG. 3A

FIG. 3B

▨ no IL-10
▨ +IL-10

α-IL-10(μg/ml)

FIG. 3C

FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

FIG. 5

FIG. 6

FIG. 7B

FIG. 7A

FIG. 8A

previous MLR
with medium   IL-10   αIL-2R

FIG. 8B

FIG. 9A

previous activation
with CD3 mAbs plus medium   IL-10   αIL-2R

FIG. 9B

FIG. 10

FIG. 11

FIG. 12

FIG. 13

FIG. 14A

Control
cells

Anergized
cells

CD3 mAbs +IL-2

CD3 mAbs

Before activation

IL-2 R α (CD25)

FIG. 14B

FIG. 15A

Time (seconds)

Ratio
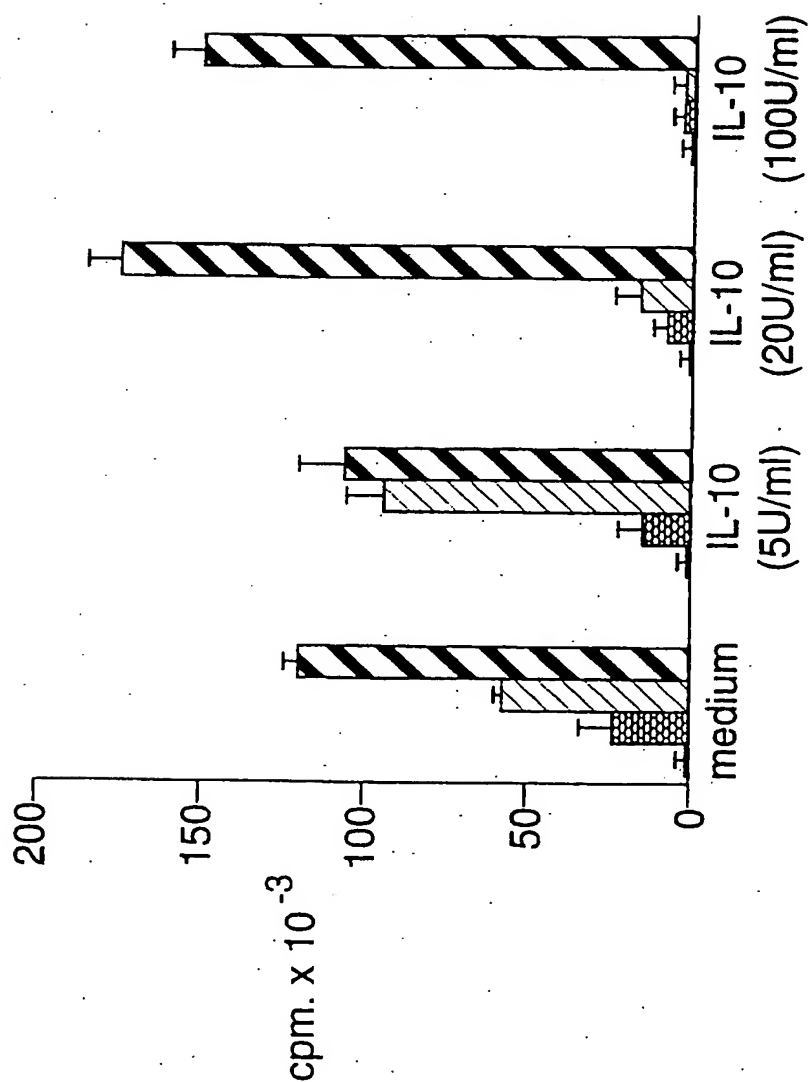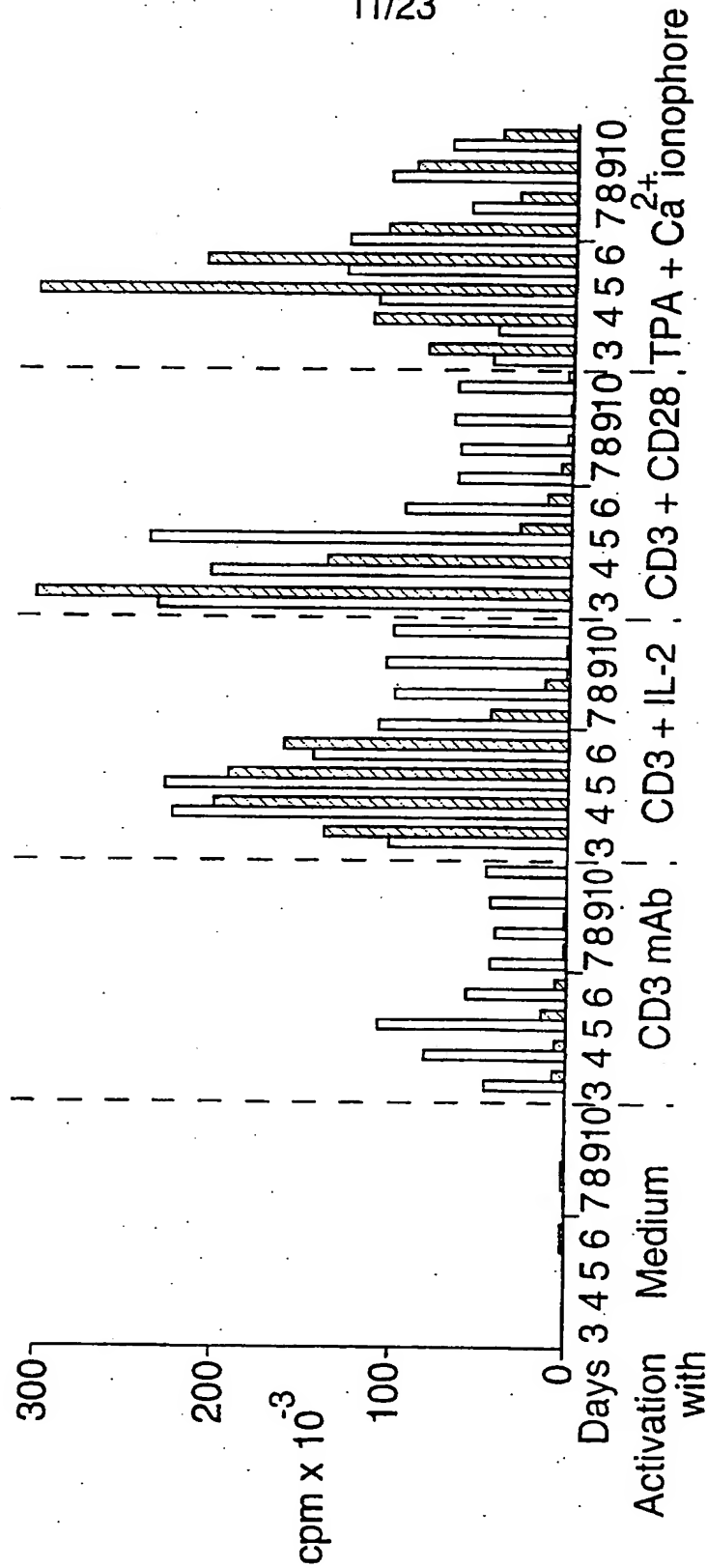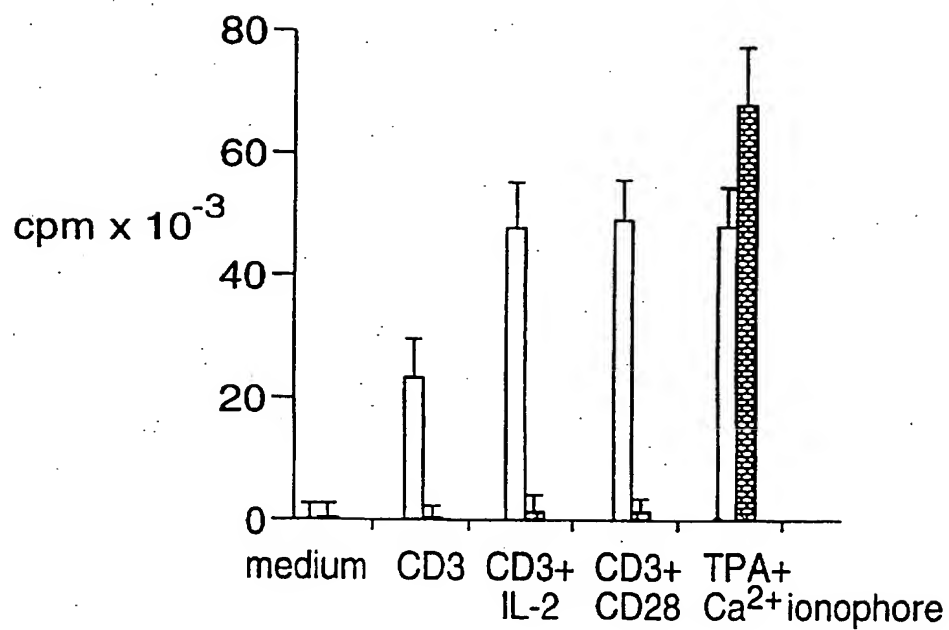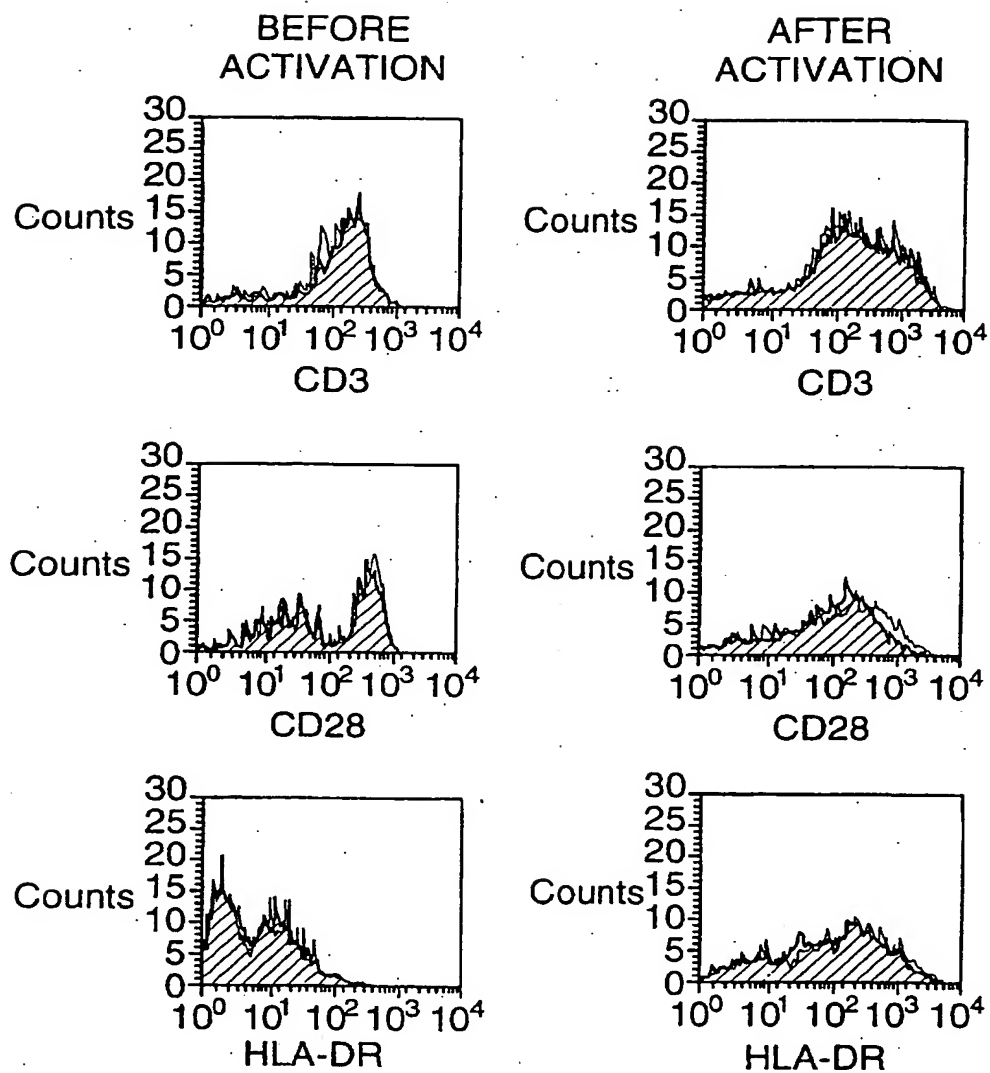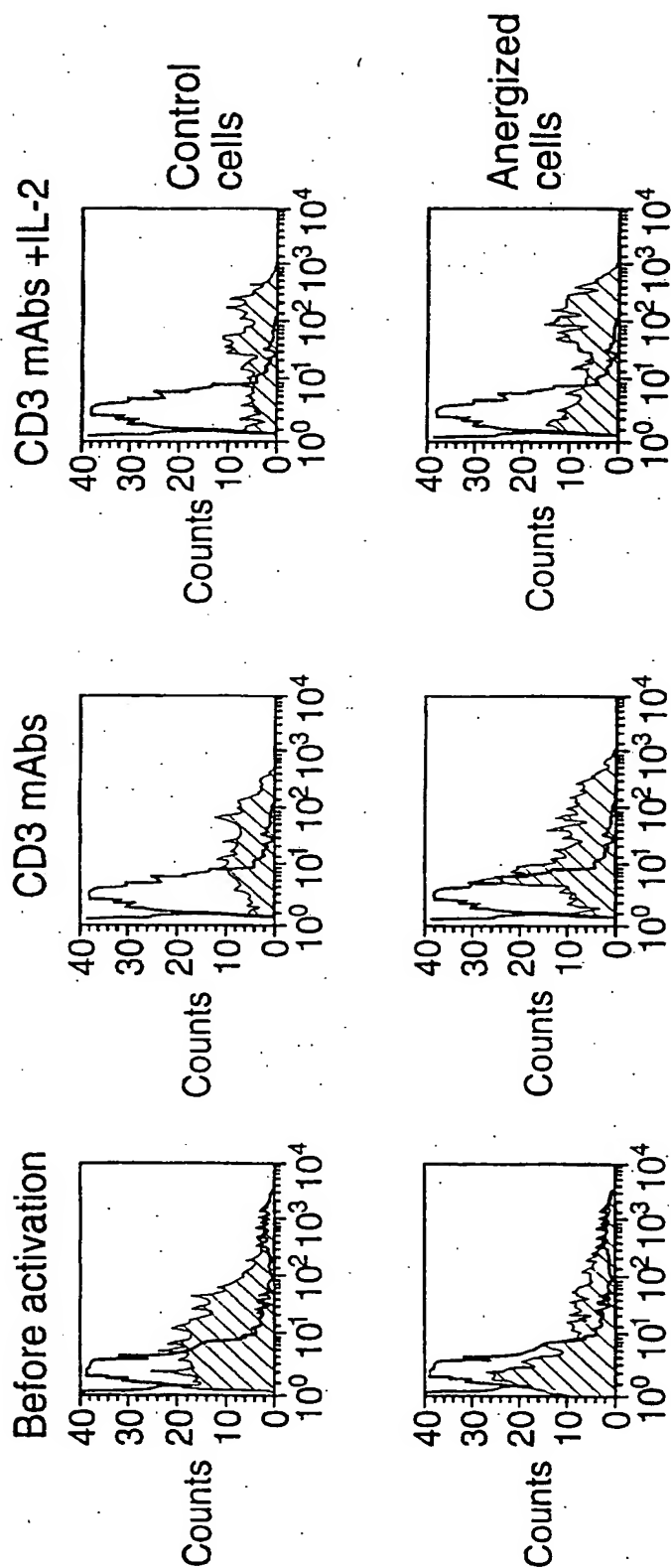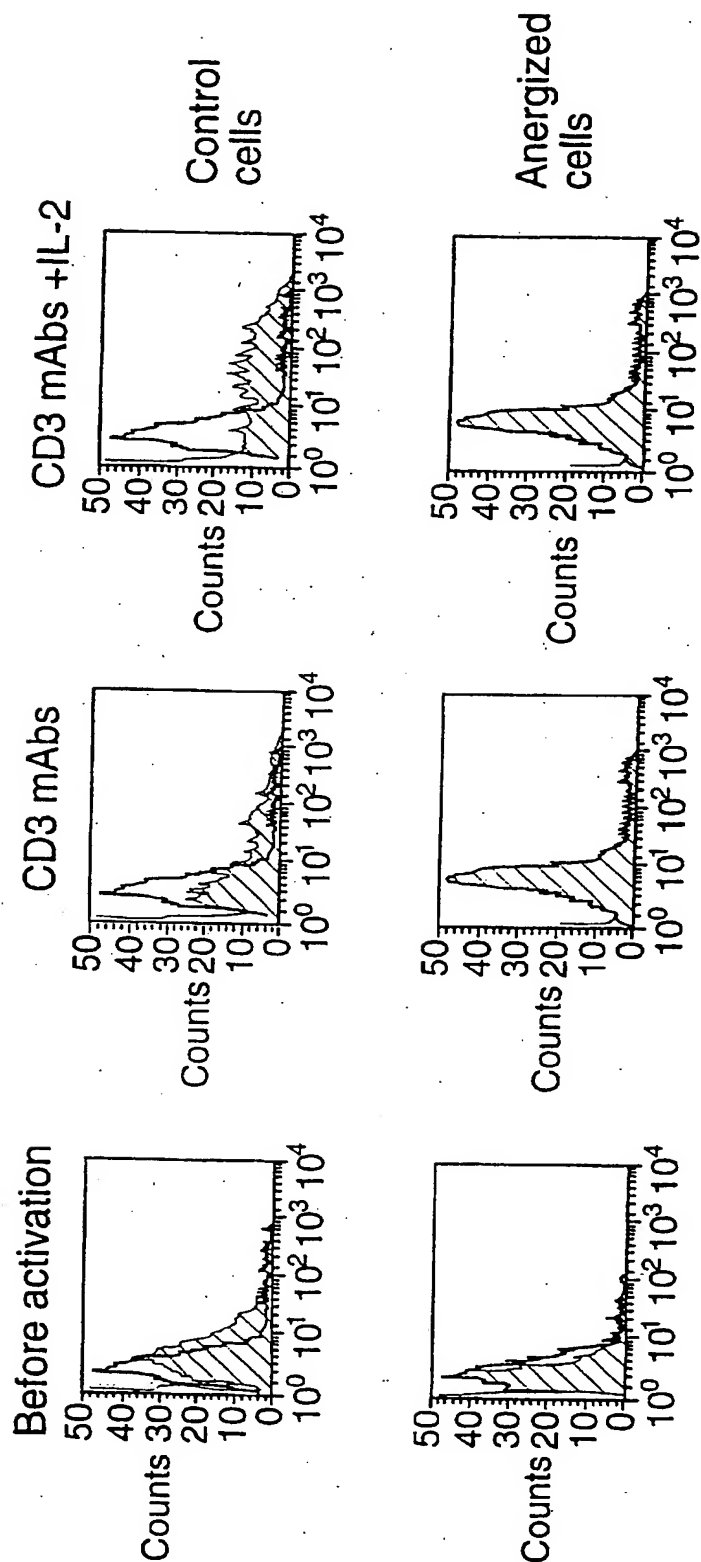
FIG. 15B

FIG. 16

Anergy can be reversed by high doses of anti-CD3 mAb

Legend:
- ⊖—— Control cells
- ⊕—— Anergic cells

Y-axis: c.p.m. x 10^3 (0, 25, 50, 75, 100)

X-axis: log dilution CD3 (ng/ml) (1, 10, 100, 1000, 10000, 100000, 1000000)
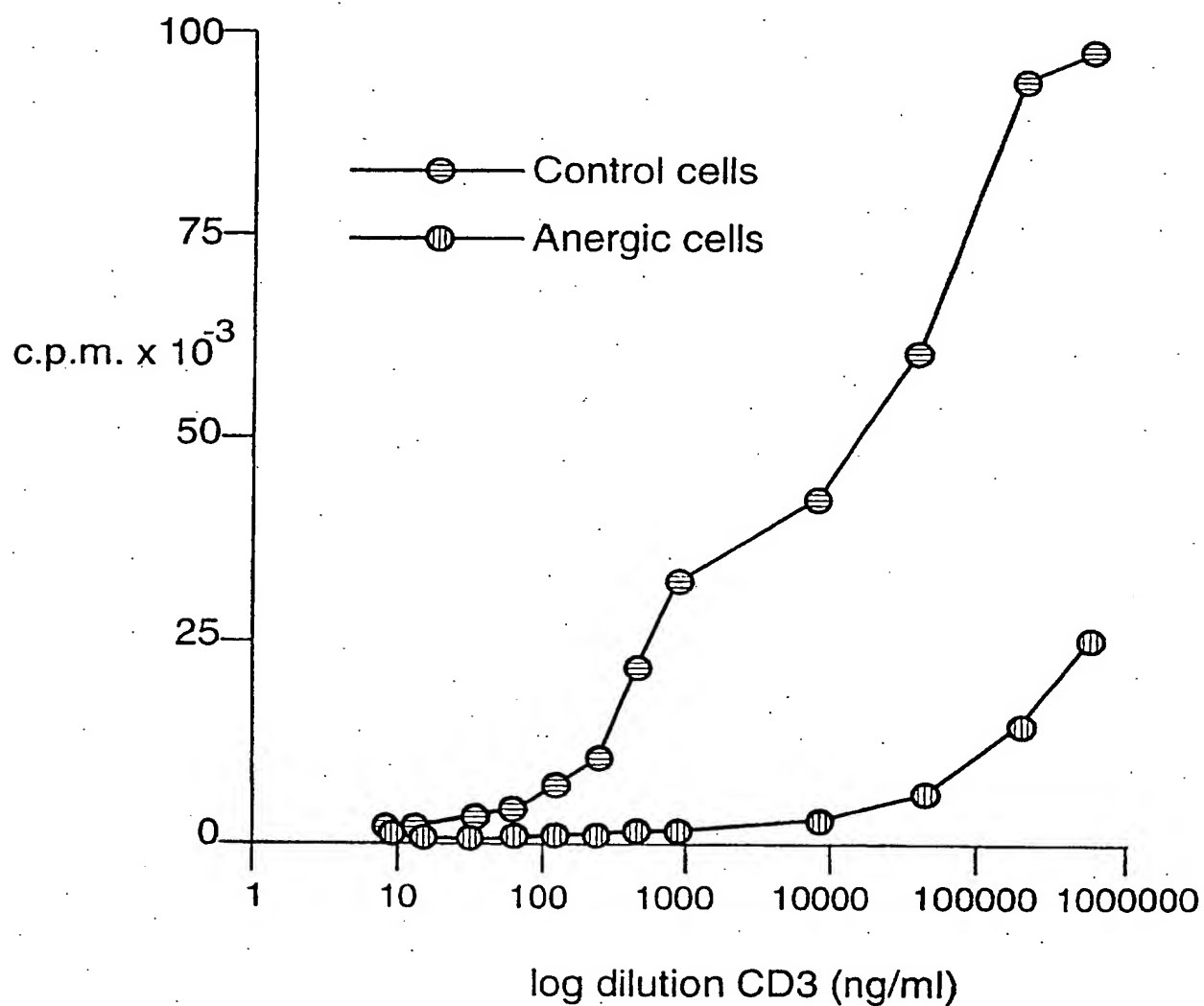
Proliferative responses of Th3 clones
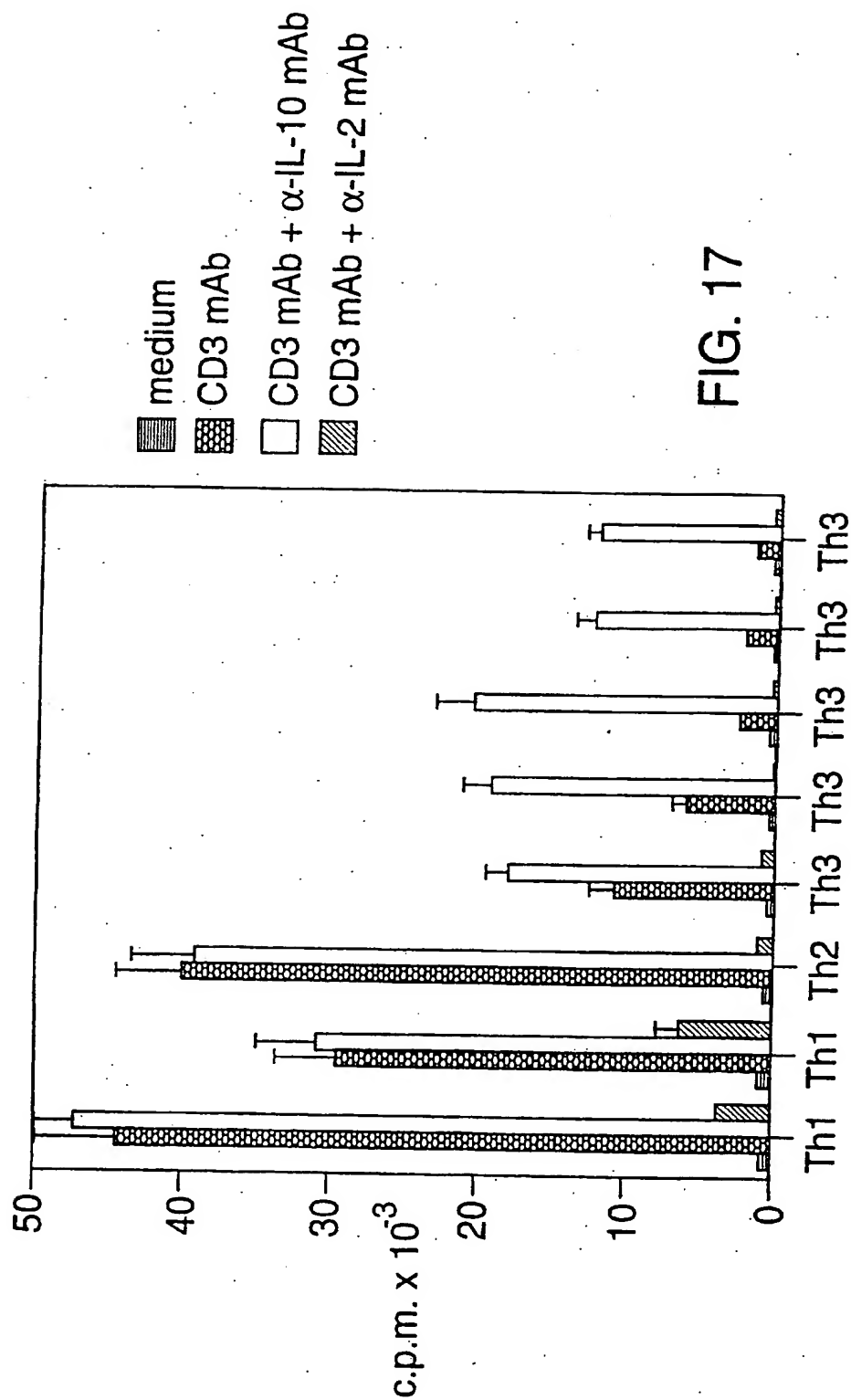
FIG. 17

FIG. 18
Kinetics of IL-10 production by Th3 clones
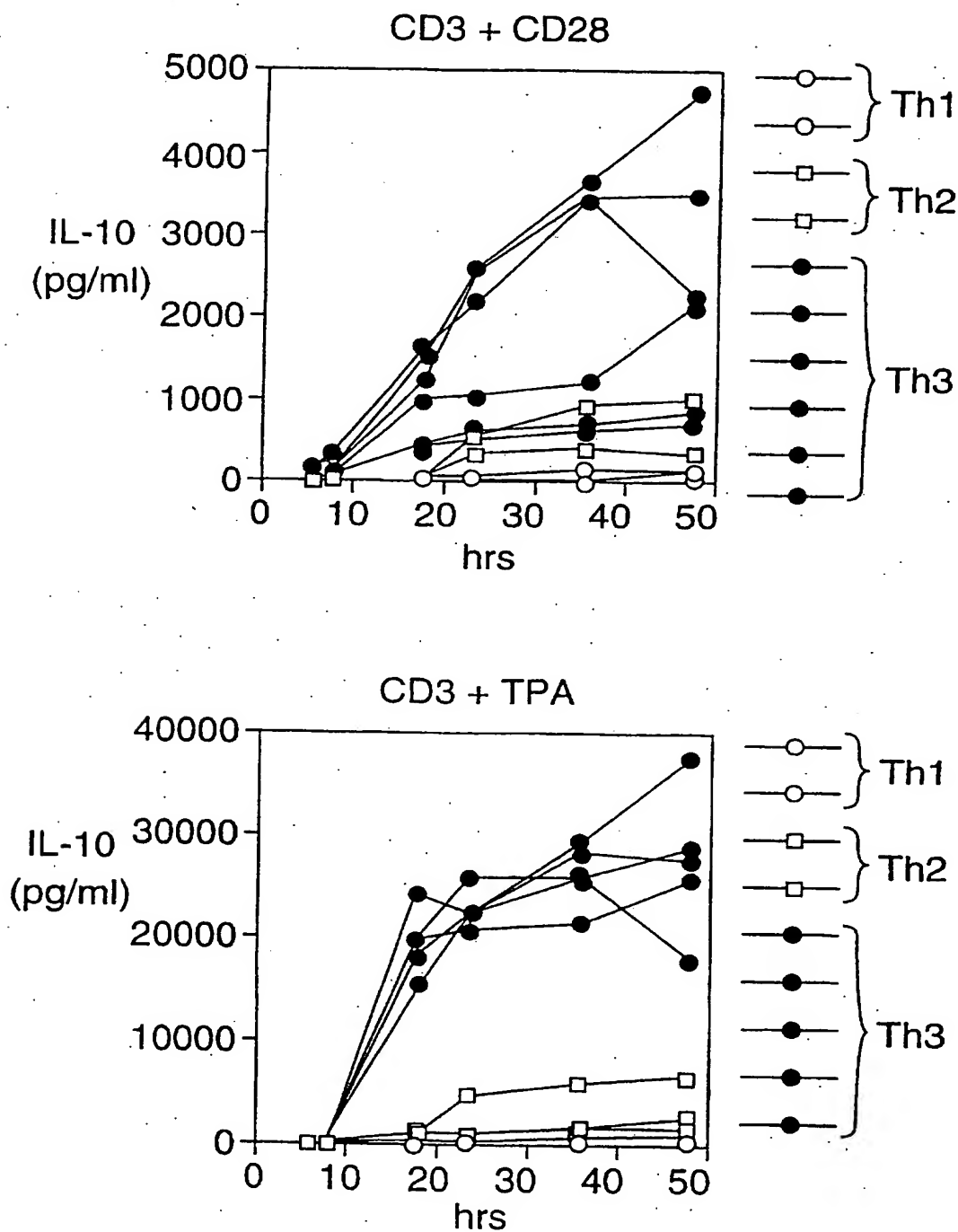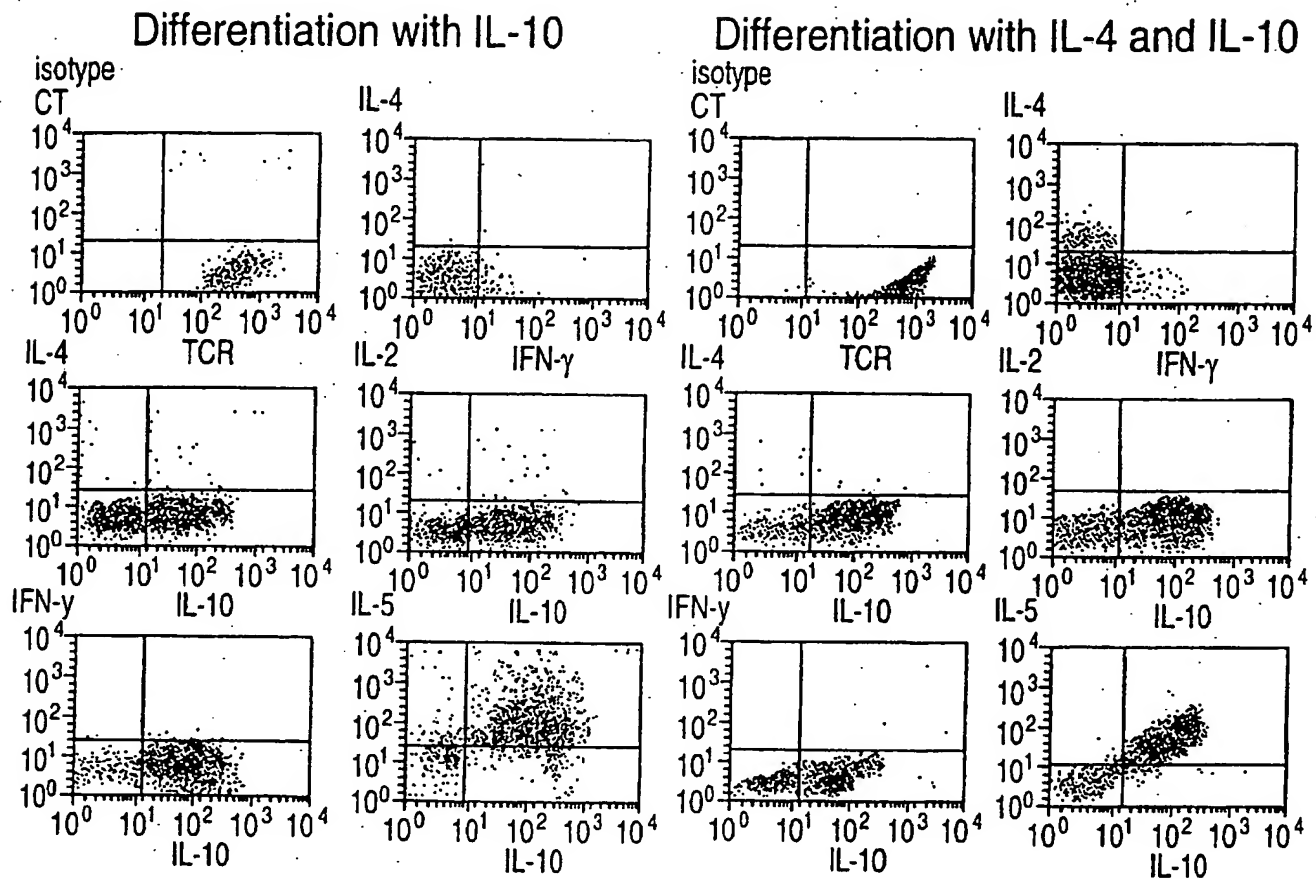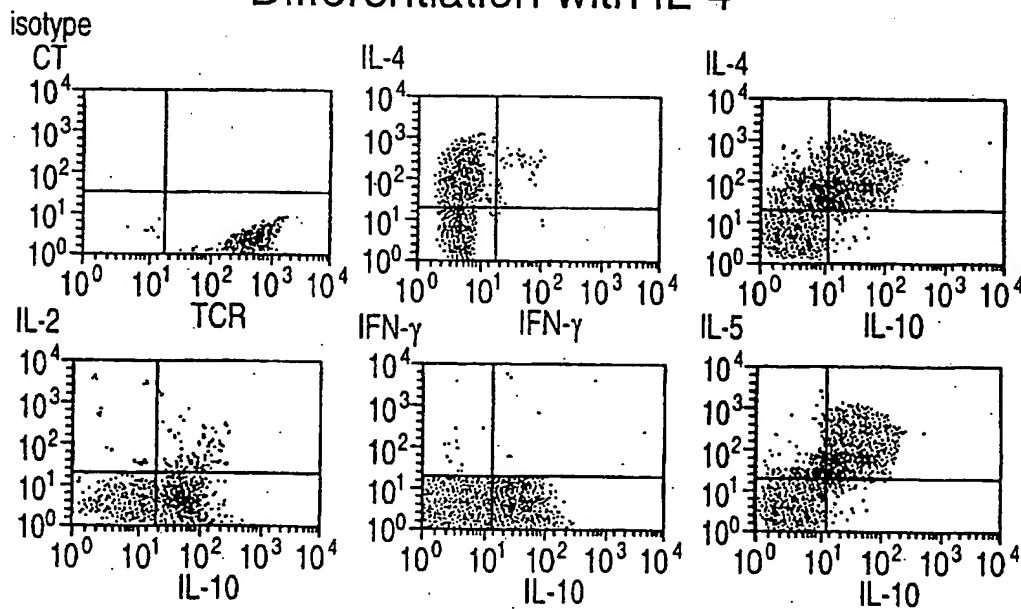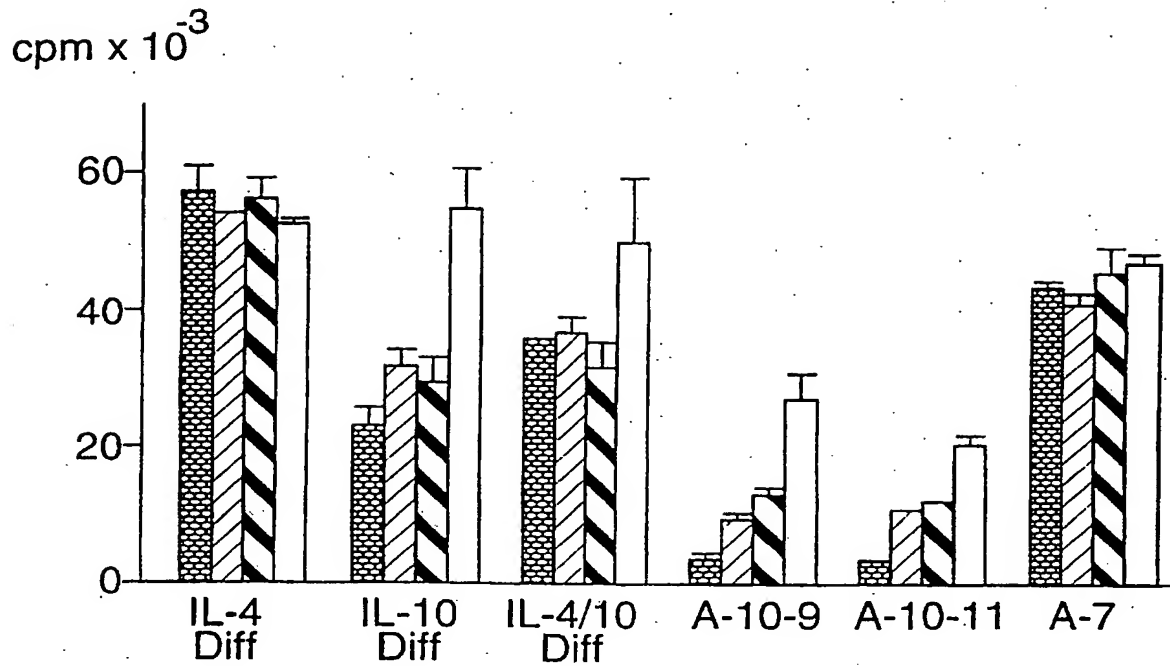
CD3 + CD28

CD3 + TPA

Differentiation with IL-10

Differentiation with IL-4 and IL-10

Differentiation with IL-4

FIG. 19

cpm x 10^{-3}

FIG. 20A

cpm x 10^{-3}

FIG. 20B

cpm x 10^{-3}

FIG. 21A

cpm x 10^{-3}
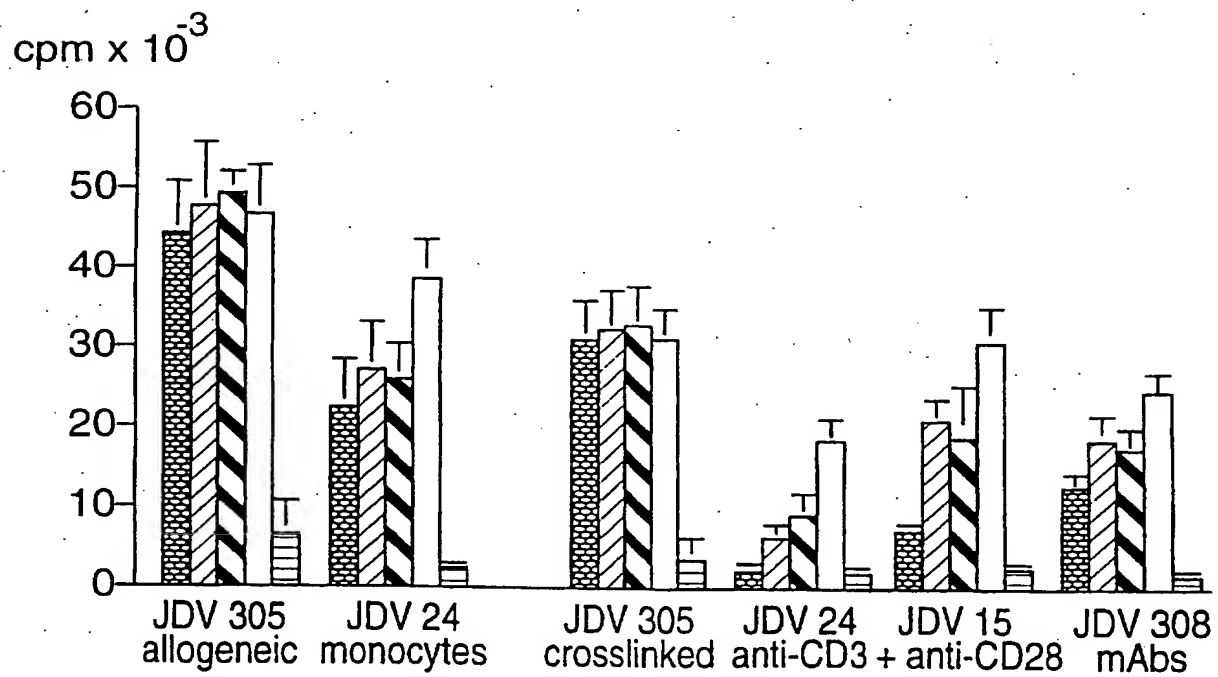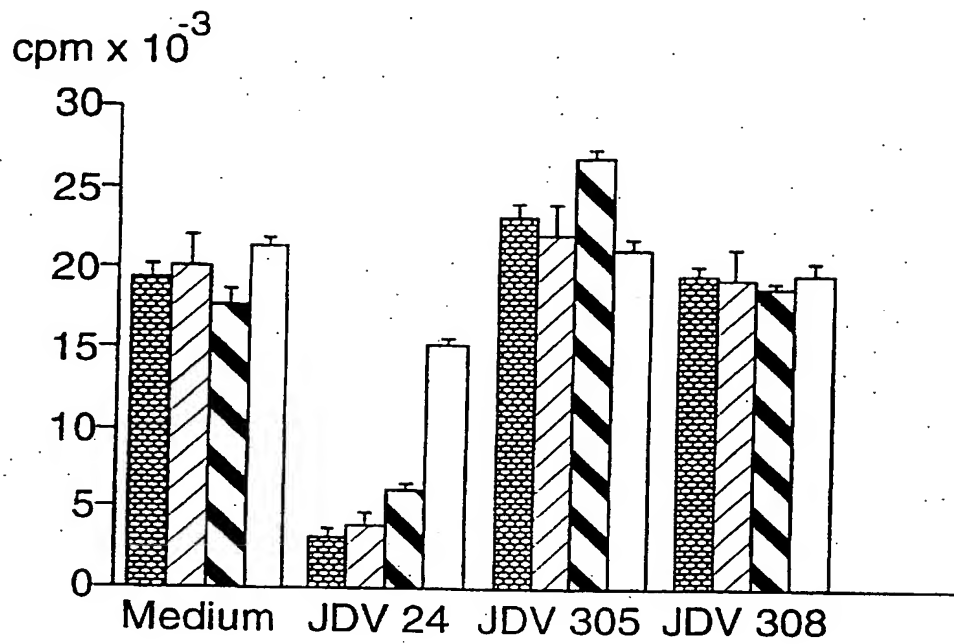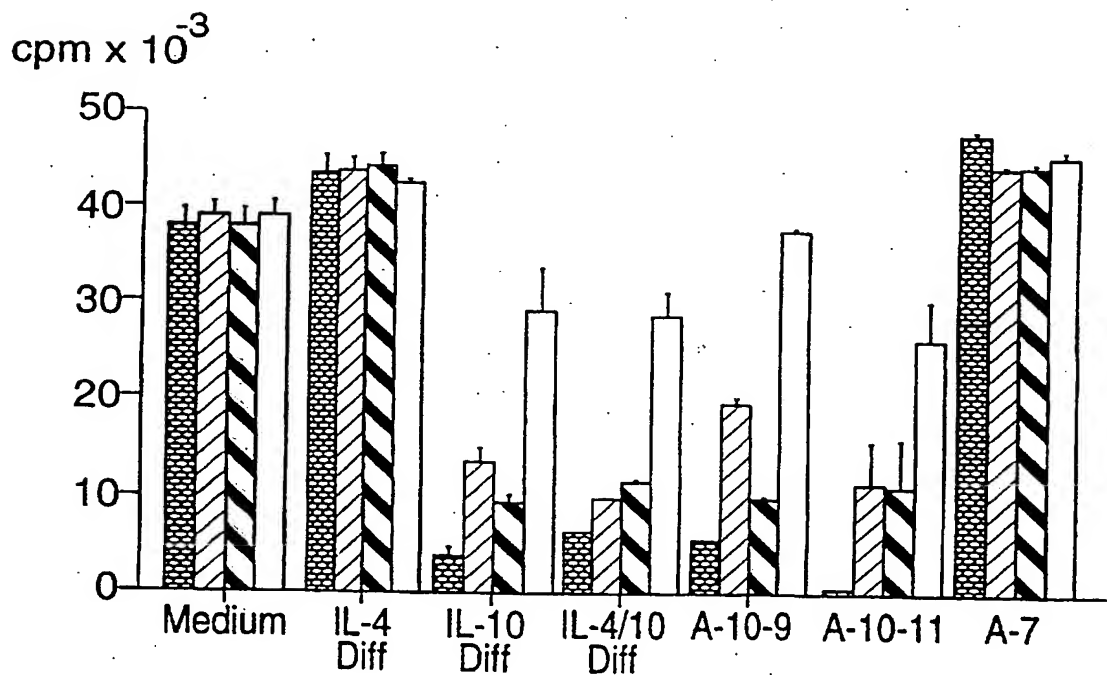
FIG. 21B

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/24   A61K38/20   C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
O,X	9TH INTERNATIONAL CONGRESS OF IMMUNOLOGY, 23 - 29 July 1995, SAN FRANCISCO,CA,USA, page 604 XP002038899 GROUX ET AL: "IL-10 INDUCES ANTIGEN-SPECIFIC ANERGY IN HUMAN PERIPHERAL BLOOD CD4+ CELLS" see abstract 3582	1-15
X	FASEB JOURNAL, vol. 10, no. 6, 30 April 1996, page A1027 XP002038900 GROUX ET AL: "IL-10 INDUCES A LONG TERM ANTIGEN-SPECIFIC STATE IN HUMAN CD4+ T CELLS" see abstract 162	1-15

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[X] Further documents are listed in the continuation of box C.   [ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
26 August 1997	11 09 97

Name and mailing address of the ISA	Authorized officer
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page 1 of 2

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF IMMUNOLOGY, vol. 154, 1995, pages 3204-3212, XP002038901 SCHOLS ET AL: "UNIQUE CYTOKINE PRODUCTION PROFILE OF ANERGIC HUMAN T CELLS IN SCID-HU MICE AFTER STAPHYLOCOCCAL ENTEROTOXIN B ADMINISTRATION" see page 3204 see abstract	
A	FASEB JOURNAL, vol. 10, no. 6, 30 April 1996, page A1444 XP002038902 WEINER ET AL: "INDUCTION AND CHARACTERIZATION OF TGF-BETA SECRETING TH3 CELLS" cited in the application see abstract 2558	
A	JOURNAL OF EXPERIMENTAL MEDICINE, vol. 179, February 1994, pages 589-600, XP002038903 POWRIE ET AL: "REGULATORY INTERACTIONS BETWEEN CD45RBHIGH AND CD45RBLOW CD4+ T CELLS ARE IMPORTANT FOR THE BALANCE BETWEEN PROTECTIVE AND PATHOGENIC CELL-MEDIATED IMMUNITY" cited in the application see page 589, abstract	
P,X	JOURNAL OF EXPERIMENTAL MEDICINE, vol. 184, no. 1, 1 July 1996, pages 19-29, XP002038904 GROUX ET AL: "INTERLEUKIN-10 INDUCES A LONG-TERM ANTIGEN-SPECIFIC ANERGIC STATE IN HUMAN CD4+ T CELLS" see the whole document	1-15

1